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Systematics and the Evolution of Calls and Mating Preferences on Túngara Frogs (Genus *Engystomops*)

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Systematics and the Evolution of Calls and Mating Preferences on Túngara Frogs (Genus *Engystomops*)

by

Santiago R. Ron, M.A.

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To Giovanna

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Systematics and the Evolution of Calls and Mating preferences in frogs of the genus *Engystomops*

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Santiago R. Ron, Ph.D.
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Sexually selected traits are among the most costly, complex, and conspicuous elements of the phenotype. In polygynous reproductive systems, they evolve under strong selection by females. Why females favor those traits, however, is an on-going debate. Here, I use túngara frogs as a model system to study the evolution of communication under sexual selection. The wealth of available information on the behavior, neurophysiology, and reproductive biology of túngara frogs make them an ideal system to understand the patterns of signal evolution and explore the processes that have shaped them. In chapter 1 and 2, I review the taxonomy of túngara frogs (*Engystomops*) from western Ecuador. I describe three new species including their external morphology and advertisement calls. In chapter 3, I explore the phylogenetic relationships of túngara frogs, testing the support for alternative relationships previously reported for this group. The new phylogeny provides the framework for the comparative analysis carried out in

chapters 4 and 5. In chapter 4, I present new female preference and male advertisement call data to test the sensory exploitation hypothesis of sexual selection. Using ancestral character reconstruction, I found that female preferences for complex calls did not originated before the appearance of complex calls, as predicted by sensory exploitation. Instead, my results suggest that the origin of complex calls and their preference originated at similar times. Finally, in chapter 5, I analyze the macroevolutionary patterns of call variation in male túngara frogs. A generalized least squares model demonstrates that advertisement calls have a strong phylogenetic signal. Although most species in *Engystomops* have distinctive calls, they share a common acoustic structure with two components that evolve at different rates. I did not found evidence of greater call differentiation among sympatric species relative to allopatric species.

Table of Contents

Chapter 1

Two New Species of *Physalaemus* (Anura: Leptodactylidae) from Western Ecuador*

Abstract. I describe two new species of the leptodactylid frog genus *Physalaemus* from the lowlands of western Ecuador. Both species belong to the *P. pustulosus* species group. They differ from other group members, except *P. coloradorum*, in their smaller size. They can be distinguished from *P. coloradorum* by their less tuberculate dorsal skin and a more depressed loreal region. The new species differ from each other markedly in advertisement call. Although the ranges overlap, body size, flank gland length, and parotoid gland length (relative to snout-vent length) are also significantly different. Overall the new species are similar in color patterns and body shape; they are difficult to diagnose from each other with morphological characters.

*Significant portions of this chapter have been previously published as Ron, Cannatella, & Coloma, 2004. Herpetologica 60:261–275. Throughout this chapter, for consistency with the original publication, the species of the *pustulosus* group are assigned to the genus *Physalaemus*. Nascimento et al. (2005) subsequently assigned these species to the genus *Engystomops* and this nomenclatural change is fallowed in Chapters 3, 4, and 5.

1.1 INTRODUCTION

THE *Physalaemus pustulosus* group has been used as a model for the study of the evolution of animal communication systems and sexual selection (Greenfield and Rand, 2000; Rand and Ryan, 1981; Rand et al., 1992; Ryan, 1980, 1985; Ryan et al., 1990; Ryan and Rand, 1990, 1993, 1995). It contains four described species: (1) *P. coloradorum* Cannatella and Duellman, 1984; (2) *P. petersi* (Jiménez de la Espada, 1872); (3) *P. pustulatus* (Shreve, 1941); and (4) *P. pustulosus* (Cope, 1864). Cannatella et al. (1998) treated *P. freibergi* Donoso Barros, 1969 (type locality "Runerrabaque, Río Beni, Bolivia") as a species distinct from *P. petersi*. Molecular and morphological synapomorphies render support to the monophyly of the group (Cannatella and Duellman, 1984; Cannatella et al. 1998).

Despite the extensive knowledge accumulated on the behavior and ecology of the group (especially *P. pustulosus*), its taxonomy is still incompletely resolved, partly because the inventories of the amphibian fauna from western Ecuador, the most speciose region for the group, are yet to be completed. Calls recorded and specimens collected in February 2002 and March 2003 have clarified the distinctiveness of two species from that region, which I describe herein.

1.2 MATERIALS AND METHODS

Morphological terminology follows Lynch and Duellman (1997). Osteological characters used in the diagnosis were examined in clear-and-stained specimens and are defined in Cannatella and Duellman (1984) and Cannatella et al. (1998). Sex was

determined by the presence of nuptial pads or by gonadal inspection. Snout-vent length is abbreviated as SVL throughout. Examined specimens (listed in the type series and Supplemental Data 1.1) are housed in the California Academy of Sciences (CAS); Museo de Zoología de la Pontificia Universidad Católica del Ecuador (QCAZ); Museum of Comparative Zoology, Harvard University (MCZ); Museum of Vertebrate Zoology, University of California Berkeley (MVZ); and Natural History Museum of The University of Kansas (KU).

Recordings were made with a Sennheiser™ ME-67 directional microphone and a Sony™ WM-D6C analog tape recorder. Calls were analyzed using Canary™ 1.2.1 software (Charif et al., 1995) at a sampling frequency of 22.1 KHz and a frequency grid resolution (FTP) = 8192. I measured nine call parameters: call length (time from beginning to end of call), rise time (time from the beginning of the call to the point of maximum amplitude), interval between calls (time between the end of one call and the beginning of the next call), duration of the first component (time from beginning of the first pulse to the end of the last pulse of the first component of the call, expressed as percentage of call length), call repetition rate (number of calls/min), fundamental frequency of the first component (frequency of the first harmonic over the duration of the first component of the call), fundamental frequency of the second component, (frequency of the first harmonic over the duration of the second component of the call), number of harmonics, and number of pulses of the first component. Original recordings are deposited in the audio archive of the QCAZ.

Shape and size of parotoid glands and flank glands were determined by making a middorsal incision to pull up the skin for inspection of its internal surface. Gland length was measured from the anterior to the posterior edge of the gland. Dorsum width was measured at the level of the insertion of the arms, perpendicularly to the dorso-ventral and antero-posterior axes, at the longest distance between the left and right margins of the dorsum. Femur length was measured from the vent to the distal edge of the flexed knee. Arm length was measured from the posterior edge of the thenar tubercle to the posterior end of the flexed elbow. Other measurements follow Duellman (1970). Measurements were made with Fowler digital calipers (nearest 0.01 mm) from specimens fixed in 10% formalin and preserved in 70% ethanol. To eliminate transcription errors, the calipers were connected to a computer and readings were automatically entered into a spreadsheet by pressing an attached foot pedal. Because of small sample sizes for females, most morphometric comparisons were only made among adult males. Only well-preserved specimens were measured (Simmons, 2002; Table 1.1). Principal Components Analysis (PCA) was applied to SVL, head length, head width, eye-nostril distance, femur length, tibia length, arm length, and dorsum width. Because of differences in body size among species, I removed the effect of size by regressing all morphological variables against SVL, retaining the residuals for the PCA (Vitt et al., 2000). Minitab 10 Xtra (Minitab, 1995) was used to perform the PCA.

Climate parameters for localities were estimated from digital climate maps compiled by C. Graham, using ArcMap 8.2 with the SpatialAnalyst extension (ESRI, 2001).

1.3 RESULTS

Physalaemus ***randi*** sp. nov.

Holotype. QCAZ 19563 (field no. SCPUCE 4885; Fig. 1.1), adult male from Ecuador, Provincia del Guayas, Cerro Masvale (2.394° S, 79.642° W), 92 m, collected by D. C. Cannatella, L. A. Coloma, A. Holloway, and S. R. Ron on 20 February 2002.

Paratopotypes. QCAZ 19558, 19560, 19563–66, 19569–71, 19573–79, 19580, 19582, 19585–88, 19590–91, 19597, 19752–55, adult males; 19570, 19583 (clear-and-stained), 19584, 19589, adult females. Collected by D. C. Cannatella, L. A. Coloma, A. Holloway, and S. R. Ron on 20 February 2002.

Paratypes. Ecuador: Provincia del Guayas: 11 km N Cerro Masvale, on the road to Virgen de Fátima (2.300° S, 79.639° W), 40 m, QCAZ 23461, 23523, adult males collected by M. R. Bustamante, I. G. Tapia, and S. R. Ron on 23 March 2003; Puerto Inca (2.536° S, 79.550° W), QCAZ 23489–92, adult males, collected by M. R. Bustamante and S. R. Ron on 20 March 2003; "10 mi E Guayaquil on road to Quito," MVZ 77184, adult male, collected by T. J. Papenfuss in 4 March 1964; El Piedrero, 15 km E from El Triunfo on the road to Pallatanga, QCAZ 11638, adult male, 11639, adult female, collected by N. Acosta-Buenaño on 21 November 1997.

Diagnosis. A member of the genus *Physalaemus* and the *P. pustulosus* group, sensu Cannatella and Duellman (1984) and Cannatella et al. (1998); see Remarks. *Physalaemus randi* is characterized by: (1) SVL 17.10 mm in males (range 15.45–18.65; $n = 35$), 18.52 mm in females (range 17.34–19.71; $n = 5$); (2) skin on dorsum bearing numerous tubercles; (3) snout rounded in dorsal and lateral views; (4) vomerine teeth and

odontophores absent; (5) maxillary teeth present; (6) parotoid glands present, mean length = 2.03 mm (n = 11, SD = 0.51; 5.8–17.0% of SVL); (7) flank glands present, mean length = 4.21 mm (n = 11, SD = 1.13; 11.7–32.1% of SVL); (8) tarsal tubercle absent; (9) nuptial pads present; (10) finger I shorter than II; (11) tympanic annulus evident, concealed dorsally, tympanic membrane not tuberculate; (12) dentigerous process of the vomer thin and spikelike.

Physalaemus randi differs from the much larger *P. pustulosus*, and *P. petersi* by the absence of a tarsal tubercle and the presence of teeth in the maxilla. *Physalaemus coloradorum* has a different advertisement call (Ryan and Rand, 2001), more tuberculated skin than *P. randi*, and a loreal region nearly vertical (slopes gradually towards the labial region in *P. randi*). *Physalaemus pustulatus* is larger than *P. randi* (non-overlapping adult size, male SVL range 25.17–29.88 mm [n = 31], and 15.45–18.65 mm, respectively) and has an advertisement call approximately twice the duration with a lower fundamental frequency (Table 1.2 and Figs. 1.2 and 1.3**)**. *Physalaemus randi* is most similar to *P. montubio* sp. nov. Both species differ markedly in advertisement call (Fig. 1.2, Table 1.2). The call of *P. randi* has longer duration (approximately three times that of *P. montubio*; Table 1.2), longer rise time (approximately five times that of *P. montubio*), lower call repetition rate (approximately half that of *P. montubio*) and a longer sequence of amplitude-modulated pulses at the beginning of the call (11–15 in *P. randi*, 2–5 in *P. montubio*). *Physalaemus montubio* is larger than *P. randi* (male mean SVL = 20.59 mm [n = 38, SD = 1.26] and 17.10[n = 35, SD = 0.74], respectively; t = 14.21, df = 71, $P < 0.0001$). *Physalaemus montubio* has proportionally shorter flank and

parotoid glands than *P. randi* (Mann-Whitney *U* for flank gland length/SVL = 54, *P* = 0.014; *U* for parotoid gland length/SVL = 51, *P* = 0.010; 11 *P. randi* vs. 21 *P. montubio*, all males; Fig. 1.4) and proportionally narrower dorsum (Mann-Whitney *U* for dorsum width/SVL = 209, *P* < 0.001; 35 *P. randi* vs. 26 *P. montubio*, all males).

Description of holotype.—Adult male, 17.55 mm SVL, foot length 8.46 mm, tibia length 7.49 mm, femur length 7.96 mm, arm length 4.08 mm, head length 5.81 mm, head width 5.40 mm, eye-nostril distance 1.83 mm, dorsum width 6.24, body wider than head; diameter of eye twice diameter of tympanic annulus; tympanic membrane and tympanic annulus barely evident; tympanic annulus ovoid, longer dorsoventrally; supratympanic fold absent; head slightly convex between orbits and flat in intercanthal region; snout rounded in profile and in dorsal view; nostrils slightly elevated, internarial region concave; canthus rostralis rounded; loreal region convex with concave depression extending from posterior border of nostril to ventral border of orbit**.**

Fingers without expanded discs; nuptial pad present, brown, divided in two portions, one covering thenar tubercle posteroventrally, other covering base of Finger I posterodorsally. Base of palmar tubercle slightly bifid, base of thenar tubercle ovoid; palmar tubercle less prominent than thenar tubercle; subarticular tubercles conical with round base; low supernumerary tubercle on Finger III between first and second subarticular tubercles; low supernumerary palmar tubercles present (Fig. 1.5). Webbing between fingers absent; relative lengths of adpressed fingers III > IV > II > I (Fig. 1.5). Toes without expanded discs; base of inner metatarsal tubercle and outer metatarsal tubercle ovoid; subarticular tubercles with round base, all subconical except for conical

proximal subarticular tubercles; flat plantar supernumerary tubercles, longitudinally aligned; tarsal tubercle absent; webbing between toes absent; relative lengths of toes IV > III > V > II > I (Fig. 1.5).

Skin on dorsum bearing numerous round tubercles, those on scapular region aligned to form a V-shaped mark with apex anteriad (Fig. 1.6); skin on venter smooth**.** Tongue longer than wide; vomerine odontophores absent. Vocal slits present, parallel to margins of mandible. Deflated vocal sac forming folds on gular region and between posterior margin of tympanum and arm insertion.

Color of holotype in preservative.—Dorsum grayish brown with irregular dark patches; interorbital and intercanthal regions brown, with darker interorbital band; dorsal tubercles slightly lighter than background; margins of inverted V on scapular region black, light gray inside; cream middorsal line from posterior half of sacral region to vent; dorsal surfaces of arms and hindlimbs brown with dark brown transversal bars on exposed surfaces. Venter cream posteriorly, with increasingly numerous minute brown spots towards throat, few spots between arms; undersides of head dark gray, becoming lighter posteriorly; cream midventral stripe from jaw margin to anterior half of abdomen, ill-defined towards end; ventral surfaces of limbs cream with numerous minute brown spots on outer edge; sides of head dark gray with two cream suborbital marks; flanks dark gray, with light brown flank glands.

Color of holotype in life.—(based on color photograph) Dorsum grayish brown with dark marks; margins of inverted V on scapular region black, light gray inside; interorbital and intercanthal regions brown; white labial line below eye and tympanum, tympanum

dark gray; dark gray flanks; arms tan orange with darker forearms; exposed surfaces of thighs brown; dark brown transversal bars in exposed surfaces of forelimbs and hindlimbs. Dark brown iris.

Etymology.—The specific name *randi* is a noun in the genitive case and is a patronym for A. Stanley Rand, who has contributed to *Physalaemus* collections in western Ecuador, and most importantly, was a pioneer in behavioral studies of *Physalaemus.* His extensive research, in collaboration with M. J. Ryan, has greatly enriched the understanding of animal communication systems.

Variation.—There is extensive variation in the dorsal coloration of preserved specimens (Fig. 1.6). Most of the variation pertains to (1) number and distribution of dark marks, (2) hue of the background coloration, varying from light gray (QCAZ 19584; Fig. 1.6) to dark gray or dark brown (QCAZ 19575), (3) abundance and arrangement of tubercles (all lighter than the background), and (4) relative longitude of the cream-dorsal line, which is continuous or interrupted and varies between being restricted to the sacral region (e.g., holotype) and almost reaching the head (e.g., QCAZ 19580). In some individuals (e.g., QCAZ 19584; Fig. 1.6), the lighter area in the occipital region, limited by rows of tubercles forming an inverted V, is absent.

Ventral surfaces of all preserved specimens have a cream background with dark markings. The color of the markings varies from light gray (QCAZ 19581) to dark gray (QCAZ 19566). In some, the markings are restricted to the head (QCAZ 19586, QCAZ 19581) or absent only in the posterolateral margins of the abdomen (QCAZ 19591). Variation between the extremes is continuous. On the abdomen, dark markings may be

arranged in well-defined large spots (QCAZ 19591) or in diffuse speckled patterns (QCAZ 19576). Relative length of the midventral cream stripe, beginning at the tip of the snout, varies from being restricted to the head (QCAZ 19580) to extending to two-thirds of the SVL (QCAZ 19589).

The lateral head coloration varies extensively between light gray and dark gray or dark brown. In the dark-brown colored QCAZ 19591, the suborbital cream stripes are thin, well defined, with the posterior stripe extended below the tympanum and the parotoid gland. In addition, two ill-defined light brown labial bars are present anteriorly. In the light gray QCAZ 19586, the two suborbital cream stripes are fused into a single wide band. In QCAZ 19580, the loreal and suborbital areas are cream except for a light brown labial stripe; the canthal region is dark brown. One individual (QCAZ 19578) has black canthal stripes.

Live specimens from 11 km N Cerro Masvale, on the road to Virgen de Fátima (QCAZ 23461, 23523) had an orange middorsal line running from the posterior half of the sacral region to the vent; the lighter zone between the dark V-shaped mark on the scapular region was also orange.

Morphometric data pertains only to adults. In the type series, the largest male has a SVL of 18.65 mm, and the largest female 19.71 mm; mean male SVL = 17.10 mm (n = 35, SD = 0.74), mean female SVL = 18.52 mm (n = 5, SD = 0.87). Snout-vent length is significantly different between the sexes (t = 3.93, df = 38, P = 0.0003). Snout-vent length is not significantly different between males of Cerro Masvale and Puerto Inca (t = 1.53, df = 31, P = 0.14). The smallest specimen of each sex in the type series (n = 40) are

the only two collected in El Piedrero, a locality 40 km E of Cerro Masvale. Descriptive statistics of morphometric measurements for two populations are given in Table 1.1.

All morphometric variables (Table 1.1) show a significant positive relation with SVL (simple regressions; ANOVA $P < 0.03$, df = 37). The relation was not significant between SVL and flank gland length ($F = 2.24$, df = 10, $P = 0.16$) and between SVL and parotoid gland length ($F = 0.05$, df = 10, $P = 0.83$). Flank gland length is not correlated with parotoid-flank length ($F = 0.41$, df = 10, $P = 0.53$). Total gland length (flank + parotoid) varies between 17.4% of SVL (QCAZ 19775 from Cerro Masvale) and 44.4% of SVL (QCAZ 19591 from Cerro Masvale). In all specimens, parotoid and flank glands are distinct from each other.

Distribution and ecology.—Physalaemus randi has been recorded in western Ecuador (Provincia del Guayas) from sea level to 150 m (Fig. 1.7). In the lowlands of western Ecuador, south of 1° latitude, precipitation is extremely seasonal, with higher precipitation in February–April (Lynch and Duellman, 1997). Among the known localities, annual precipitation ranges between 1031–2202 mm with only 0–45 mm during the three driest months of the year (July–September at Cerro Masvale); mean annual temperature ranges between 24.4 and 25.8 C (El Piedrero and 10 miles E from Guayaquil, respectively). Cerro Masvale is part of a private natural reserve adjacent to a national protected area, Reserva Ecológica Manglares-Churute.

Localities are in the following vegetation types (as defined by Cerón et al., 1999): Lowland Deciduous Costa Forest (10 miles E from Guayaquil, 11 km N Cerro Masvale, Puerto Inca), Lowland Semideciduous Costa Forest (Cerro Masvale), and Lowland

Evergreen Costa Forest (El Piedrero). All individuals collected in 2002 were found in artificial open areas. At Cerro Masvale, frogs were breeding in the vicinities of buildings, pastures, and agricultural lands. At 11 km N Cerro Masvale males were calling from a flooded rice field. At Puerto Inca males were calling from flooded grassland next to a banana plantation.

Reproductive activity is nocturnal. Choruses of males were found in Cerro Masvale, 11 km N Cerro Masvale, and Puerto Inca (20 February 2002, 21–25 March 2003) during the rainy season. Males call from small ponds and ditches while floating in a few centimeters of water, usually concealed by vegetation. Amplexus and egg deposition take place at the same sites where choruses call. As in most other Leptodactylinae, *P. randi* constructs floating foam nests. The nest is constructed during amplexus: while the female deposits the egg masses, the male beats them with his legs. At some ponds at Cerro Masvale, calling males were abundant (>1 individual/m^2), and some of them called within 20 cm of each other.

Call.—One male in a chorus at Cerro Masvale (QCAZ 19752, call QCAZ (S) 19752; SVL = 17.23 mm) was recorded at 2025 h on 20 February 2002 while calling next to a ditch, partly submerged in 0.5 cm of water, among vegetation (water temperature 25.4 C, air temperature 24.8 C). The call is a single note with up to six harmonics. The note has two components: a sequence of 14 to 15 amplitude-modulated pulses followed by a nearly pure tone "whine" with a downward frequency sweep (Fig. 1.2). Both components have approximately the same duration (first component lasts on average 49.7% of the call; range 44.5–56.0, n = 17). The fundamental frequency of the first

component is higher than that of the second component. In a sequence of 17 calls, the dominant frequency of the first component is either in the first harmonic (*n* = 9, mean frequency = 1.314 KHz) or the second (*n* = 8, mean frequency = 2.885 KHz). In the second component, the dominant frequency is always the first harmonic. Each note sweeps downward in frequency by about 0.5 KHz. Call parameters for QCAZ 19752 are shown in Table 1.2. Similar call structure was recorded for QCAZ 23462 (call QCAZ (S) 23462, SVL = 18.76 mm) at the same locality on 21 March 2003 at 2025 h (water temperature 27.9 C, air temperature 27.5 C; Table 1.2). However, in this individual the dominant frequency is always the fundamental for both components. The first component lasts on average 50.0% of the call (range 44.4–58.0, *n* = 16).

The advertisement call of *P. randi* is shorter and has a higher frequency than that of *P. pustulatus*. The call of *P. pustulatus* consists of a frequency-modulated tone, similar to the "whine" component of the call of *P. pustulosus* (Fig. 1.3; Ryan, 1985). Call parameters for *P. pustulatus* (Figure 1.3, Table 1.2) were measured from a recording of a male in a chorus at Puerto Rico (QCAZ 19518, call QCAZ (S) 19518; SVL = 25.85 mm) at 2040 h in 17 February 2002. The male was calling partly submerged in water (water temperature 26.8 C, air temperature 24.0 C).

Remarks.—Physalaemus randi is assigned to the *P. pustulosus* group based on the presence of four synapomorphies (Cannatella et al., 1998): (1) presence of flank glands, (2) presence of parotoid glands, (3) warty skin, and (4) dentigerous process of the vomer thin and spikelike.

Physalaemus ***montubio*** sp. nov.

Holotype.—QCAZ 19524 (field no. SCPUCE 4820), an adult male from Ecuador, Provincia de Manabí, Puerto Rico (1.639° S, 80.830° W), 30 m, collected by D. C. Cannatella, L. A. Coloma, A. Holloway, and S. R. Ron on 18 February 2002.

Paratopotypes.—QCAZ 19375–80, 19511, 19515–17, 19519–22, 19520–22, 19526–27, 19530–33, 19549–50, 19552, 19555–57, adult males; 19512, 19525, adult females, collected by D. C. Cannatella, L. A. Coloma, A. Holloway, and S. R. Ron on 17–18 February 2002.

Paratypes.—Ecuador: Provincia de Manabí: Río Cuaque, in the road to Recinto 10 de Agosto (0.014° S, 80.073° W), 20 m, QCAZ 19383–86, adult males, collected by F. Ayala on 15 February 2002; Río Cuaque, in the road between Pedernales and El Carmen, 100 m, KU 218219, adult female, collected by D. Kizirian, F. Campos, J. J. Wiens, and L. A. Coloma on 31 December 1989; Puerto Cayo, QCAZ 14729, adult male, collected by R. Gattelli on 9 February 2000; Río Chico near Salango, QCAZ 12340–41, 12362, adult males, 12339, adult female, collected by F. Campos and M. J. Barragán on 4 July 1998; Calceta, QCAZ 3728, adult female, collected by G. Onore on 2 February 2002.

Diagnosis.—A member of the *P. pustulosus* group as defined by Cannatella et al. (1998); see Remarks. *Physalaemus montubio* is characterized by (1) mean SVL 20.59 mm in males (18.67–22.56; $n = 38$), 22.62 mm in females (SVL 21.58–24.16; $n = 4$); (2) skin on dorsum bearing numerous round or subconical tubercles; (3) snout subacuminate in dorsal view, rounded in profile; (4) vomerine odontophores absent; (5) maxillary teeth present; (6) parotoid glands present, mean length = 1.96 mm (SD = 0.65; 5.0–16.6% of SVL; $n = 20$) ; (7) flank glands present, mean length = 3.6 mm (SD = 1.8; 4.5–31% of

SVL; *n* = 20); (8) tarsal tubercle absent; (9) nuptial pads present; (10) finger I shorter than II; (11) tympanic annulus evident, concealed dorsally; tympanic membrane not tuberculate; (12) dentigerous process of the vomer thin and spikelike; (13) stalk of the alary process of the hyoid very narrow; (14) anterior process of hyale well developed and prominent.

Physalaemus montubio (Fig. 1.8) differs from the larger *P. pustulosus*, and *P. petersi* by the absence of a tarsal tubercle and the presence of teeth on the maxilla and premaxilla. *Physalaemus coloradorum* has a different advertisement call (Ryan and Rand, 2001), more tuberculated skin than *P. montubio*, and a nearly vertical loreal region (slope gradually towards the lips in *P. montubio*). The ranges of adult size of *P. montubio* and *P. pustulatus* do not overlap (male SVL range 18.67–22.56 mm and 25.17–29.88 [*n* = 31], respectively). Additionally, *P. pustulatus* has an advertisement call approximately five times longer with a lower dominant frequency (Table 1.2, Figs. 1.2 and 1.3**)**. *Physalaemus montubio* is most similar to *P. randi*. The species differ markedly in at least four parameters of their advertisement calls (i.e., call duration, call repetition rate, rise time, and number of pulses at the beginning of the call; see *P. randi* diagnosis for details; Table 1.2, Fig. 1.2). *Physalaemus montubio* has a greater SVL and proportionally shorter flank and parotoid glands than *P. randi* (Fig. 1.4; see *P. randi* Diagnosis).

Description of holotype.—Adult male, 22.55 mm SVL, foot length 10.91 mm, tibia length 10.57 mm, femur length 10.46 mm, arm length 4.91 mm, head length 7.43 mm, head width 7.82 mm, eye-nostril distance 2.35 mm, dorsum width 8.77 mm, body as wide as head except on scapular region where it is 1.3 mm wider; diameter of eye 2.2 times the

diameter of tympanic annulus; tympanic membrane and tympanic annulus barely evident; tympanic annulus rounded; supratympanic fold absent; head slightly convex between orbits and intercanthal region; snout rounded in profile and in dorsal view; nostrils slightly elevated anteriorly, internarial region concave; canthus rostralis rounded; loreal region convex with concave depression from posterior border of nostril to anteroventral border of orbit.

Fingers without expanded discs; nuptial pad present, brown, covering posterior two thirds of thenar tubercle and extending to base of thumb (Fig. 1.5). Base of palmar tubercle ovoid and slightly pointed anteriorly, smaller than base of ovoid thenar tubercle; subarticular tubercles conical with round base; flat supernumerary palmar tubercles present. Webbing between fingers absent; relative lengths of adpressed fingers III > IV > II > I (Fig. 1.5). Toes without expanded discs; base of inner metatarsal tubercle ovoid, larger than ovoid base of outer metatarsal tubercle; inner metatarsal tubercle more prominent than outer metatarsal tubercle; subarticular tubercles with round base, all conical except for subconical distal subarticular tubercles of fingers III, IV, and V; flat plantar supernumerary tubercles, longitudinally aligned; tarsal tubercle absent; webbing between toes absent; relative lengths of toes IV > III > V > II > I (Fig. 1.5).

Skin on dorsum bearing numerous, minute, round tubercles, more abundant in sacral region; skin on venter smooth. Tongue longer than wide; vomerine teeth and odontophores absent. Vocal slits present, parallel to margins of mandible. Deflated vocal sac forming folds on gular region and extending posteriorly to proximal end of the arm.

Color of holotype in preservative.—Dorsum grayish brown with irregular dark patches; two faint dark longitudinal stripes from sacral region to posterior edge of orbits; dorsal tubercles lighter than background; light gray middorsal line from posterior two-thirds of sacrum to vent; dorsal surfaces of forearms and hindlimbs brown with dark brown transversal bars on exposed surfaces, arms light brown. Venter cream posteriorly with few scattered minute brown spots; brown blotches present on anterior half of venter except on proximity of armpits; ventral surfaces of head dark brown; light brown medial stripe from jaw tip to scapular region; ventral surfaces of hindlimbs and arms cream with numerous minute brown spots on outer edge, ventral surfaces of forearms brown becoming cream towards the inner edge; sides of head brown with light brown area below the orbit and tympanum; flanks dark gray ventrally, light gray dorsally.

Color in life.—(KU 218219, adult female from Río Cuaque). Dorsum light brown with darker blotches on sides and on limbs. Venter off-white near abdomen with light brown speckling. Pelvic region, throat, and underside of legs not pigmented. Iris bronze. Dark brown stripe posterior to eye. Upper lip white. (L. A. Coloma field notes, 31 December 1989).

Etymology.—The specific name *montubio* is a noun in apposition derived from the Ecuadorian word "montubio" that refers to the people who inhabit the country side of the lowlands of western Ecuador. Because of its tolerance of habitat disturbance, *Physalaemus* are well known by montubios who refer to them as "ranas bullangueras" (noisy frogs) due to their loud advertisement calls.

Variation.—There is continuous variation in the number and shape of the dorsal tubercles (Fig. 1.9). The tubercles can be scattered and predominantly rounded (QCAZ 19520) or abundant and subconical (QCAZ 19530). The background coloration varies from light gray (QCAZ 12341,19555) to dark gray or dark brown (QCAZ 19532). A lighter middorsal band is present in some individuals (QCAZ 19512, QCAZ 19522). Dorsolateral light-gray bands are present in QCAZ 19557. There is extensive variation in the number and distribution of dark marks. Representative states within the variation of these continuous characters are shown in Figure 1.9.

Ventral surfaces of preserved specimens have a cream background with dark markings. Coloration varies mainly in: (1) the hue of the markings, varying from light gray (QCAZ 19512) to dark brown (QCAZ 19516); and (2) the distribution of the markings, which can be almost completely restricted to the head (QCAZ 19520) or the chest (QCAZ 12339) or present in all the venter (QCAZ 19516). Variation between extreme states is continuous. In the abdomen, dark marks can be arranged in well-defined large spots (QCAZ 19555) or in diffuse speckled patterns (QCAZ 14729). In all specimens (except QCAZ 12362 and 19519) a midventral cream to light brown stripe, starting on the head, is present. The extent of the stripe varies from being restricted to the head (QCAZ 19522) to reaching up to 2/3 of SVL (QCAZ 12340). The stripe varies from discrete (QCAZ 12340) to ill defined (QCAZ 19525).

Lateral head coloration varies extensively between light gray (QCAZ 12339) to dark gray or dark brown (QCAZ 19532). In specimens with darker coloration, the suborbital cream stripes are short and ill defined. In specimens with lighter coloration, a

single cream wide band is present below the orbit. One or two additional cream to light brown labial bars may be present anteriorly (QCAZ 19549).

In the type series, the largest male has a SVL of 22.56 mm, and the largest female, 24.16 mm; male mean SVL = 20.59 mm (n = 38, SD = 1.26), female mean SVL = 22.62 mm (n = 4, SD = 1.14). Small sample size of females prevents analysis of intersexual differences in size. However, available data suggest that females are larger than males. The smallest female (SVL 21.58 mm, QCAZ 19525) is larger than 80% of the males (n = 38). Among 18 adult males, combined gland length (flank + parotoid) varies from 13.7% of SVL (QCAZ 19530 from Puerto Rico) to 44.9% (QCAZ 19386 from Río Cuaque). In all specimens, parotoid and flank glands are distinct from each other.

Distribution and ecology.—Physalaemus montubio has been recorded in western Ecuador (Provincia de Manabí) from sea level to 200 m (Fig. 1.7). At the known localities, annual precipitation ranges between 156–1115 mm (Río Chico and Calceta, respectively) with only 0–33 mm falling during the three driest months of the year (Puerto Rico and Calceta, respectively; at Puerto Rico, the driest months are September–November); mean annual temperature is 24.7–26 C (Río Cuaque and Calceta).

Localities are in the following vegetation types (as defined by Cerón et al., 1999): Lowland Evergreen Costa Forest (Río Cuaque), Lowland Semideciduous Costa Forest (Calceta), and Lowland Dry Shrub (Puerto Cayo, Puerto Rico, and Río Chico). All individuals collected in 2002 were found by night in artificial open areas.

Reproductive activity is nocturnal. In Puerto Rico, frogs were breeding near buildings, pastures, and in other cleared areas. Choruses were heard in March 1999,

February 2002, and March 2003, during the rainy season. Most individuals were congregated around small temporary pools that filled after heavy rains. Males called while floating in a few centimeters of water. At Puerto Rico, a large aggregation was found in a stream pool 50 m away from the Pacific Ocean. Dozens of males were calling among aquatic plants. Interestingly the chorus was synchronized into two antiphonal "voices." Other species calling and breeding on the same pond were *Bufo marinus* and the hylids *Trachycephalus jordani* and *Scinax quinquefasciatus*. Amplexus and egg deposition take place at the same sites where choruses call. Amplectant pairs were observed to make floating foam nests. To do so, the male beats the egg masses with his legs as they are discharged by the female.

In 1999 and 2002, *P. montubio* was breeding syntopically with *P. pustulatus* at Puerto Rico. At some pools, males of both species were calling simultaneously. Coloma and Ron (2001) published a color photograph of a calling male of *P. montubio* from Puerto Rico.

Call.—One male (QCAZ 19517, call QCAZ (S) 19517; SVL = 20.85 mm) in a chorus at Puerto Rico, was recorded at 2025 h on 17 February 2002 while calling partly submerged in 2 cm of water, on a roadside pool (water temperature 27.4 C, air temperature 24.2 C). The call consists of a single, short note that begins with two to five amplitude-modulated pulses followed by a nearly pure tone with a downward frequency sweep (Fig. 1.2, Table 1.2). The mean duration of the first component is 18.6% of the call (range 11.7–24.9, $n = 13$). The call has a rich harmonic structure (Fig. 1.2). In the second component of the call, the dominant frequency is always in the first harmonic. In the

pulsed component, the dominant frequency is either the first harmonic (mean frequency = 1.369 KHz, *n* = 6) or the second (mean frequency = 3.098 KHz, *n* = 7). The holotype, QCAZ 19524, (call QCAZ (S) 19524; SVL = 22.55 mm) was recorded at 0005 h on the same night and locality. The call is similar to that of QCAZ 19517 but with more discrete pulses at the beginning of the call. In the pulsed component of the call, the dominant frequency is always in the second harmonic (mean frequency = 2.689 KHz, *n* = 15). The mean duration of the first component is 25.1% of the call (range 20.6–31.7, *n* = 15). Other call parameters are shown in Table 1.2.

Remarks.—Physalaemus montubio is assigned to the *P. pustulosus* group based on the presence of four synapomorphies (Cannatella et al., 1998): (1) presence of flank glands, (2) presence of parotoid glands, (3) warty skin, and (4) dentigerous process of the vomer thin and spikelike.

Morphometric comparisons between P. randi, *and* P. montubio.—Measurements of eight morphometric variables are given in Table 1.1. In the PCA, the first three principal components account for 59.3% of the variation (Table 1.3). Principal Component I describes a gradient based mainly on tibia and femur length. Principal Component II describes a gradient based on arm length and dorsum width. Overall, *P. montubio* and *P. randi* overlap widely in morphometric space (Fig. 1.10).

1.4 DISCUSSION

According the phylogeny presented by Cannatella et al. (1998), the *P. pustulosus* group is supported by at least four morphological synapomorphies: (1) presence of flank glands, (2) presence of parotoid glands, (3) warty skin, and (4) dentigerous process of the

vomer thin and spikelike. *Physalaemus montubio* and *P. randi* have not been included in any phylogenetic analysis previously. Nevertheless, its inclusion in the group is supported by the presence of all these morphological synapomorphies.

Two basal clades are defined in the Cannatella et al. (1998) phylogeny, one distributed in Central America, northern South America, and the Amazon basin (containing *P. petersi*, and *P. pustulosus*) and the other in western Ecuador and northwestern Peru (containing all remaining species of the group). Synapomorphies for the latter clade are: (1) absence of a tarsal tubercle, (2) narrow stalk of the alary process of the hyoid, and (3) insertion of petrohyoideus anterior muscle along edge of hyoid plate (Cannatella et al., 1998). Although character (3) has not been assessed in *P. montubio* and *P. randi*, characters (1) and (2) indicate their inclusion in the northwestern South American clade.

Numerous publications on sexual selection and call evolution of the *P. pustulosus* group have included species from western Ecuador (e.g. Rand et al., 1992; Ryan and Rand, 1990, 1993, 1995; Ryan et al., 2003). In all those accounts (except Ryan, 1990) and in Cannatella et al. (1998) there has been confusion on the assignment of specimens and calls to *P. pustulatus*. The confusion probably arose from the poor condition of the type material of *P. pustulatus*. The holotype (MCZ 7666 from "Guayaquil") was collected in 1913 and has a SVL of 20.77 mm. The internal organs had been removed, and the frog lacks nuptial pads and vocal slits. The holotype closely resembles larger *P. pustulatus* specimens from Guayaquil (KU 154561–62) indicating that it is a conspecific juvenile. Because of the condition of the holotype, much larger specimens of

Physalaemus pustulatus collected in Portoviejo (Provincia de Manabí, Cannatella et al., 1998) were mistakenly referred to an undescribed species, frequently referred as *P.* sp. C in the literature (e.g., Cannatella et al., 1998; Tárano and Ryan , 2002). *Physalaemus pustulatus* has also been recorded in Puerto Rico (Provincia de Manabí, QCAZ 19355), Patricia Pilar (Provincia de Los Ríos, QCAZ 19607), Isla Puná (Provincia del Guayas, CAS 5408), and Cerro Blanco (Provincia del Guayas, QCAZ 23427). It is not established yet if morphologically similar specimens from northwestern Peru refered as "*P. caicai*" by Ryan and Rand (2001) or "*P.* sp. B" by Cannatella et al. (1998) deserve to be considered a separate species from *P. pustulatus*. Conversely, calls and specimens from southern Ecuador (Provincia de El Oro, Pasaje) though to belong to *P. pustulatus* (e.g., Ryan, 1996; Ryan and Rand, 2001) belong either to *P. randi* or to a closely related undescribed species. Further analysis of molecular and morphological data is necessary to determine their taxonomic status.

Table 1.1. Descriptive statistics for morphometric measurements of *Physalaemus montubio* from Puerto Rico (Provincia de Manabí) and *P. randi* (from Cerro Masvale and Puerto Inca, Provincia del Guayas). Mean ± SD are given with range below. Bold figures are combined for males of both populations of *P. randi*. Abbreviations are SVL = snout-vent length; DW = dorsum width; TL = tibia length; FL = femur length; AL = arm length; HL = head length; HW = head width; EN = eye-nostril distance. All measurements are in mm.

	SVL	DW	TL	FL	AL	HL	HW	EN
P. montubio	20.76 ± 0.99	7.60±0.44	9.74 ± 0.53	9.41 ± 0.64	4.95 ± 0.33	6.58 ± 0.35	6.93 ± 0.34	2.13 ± 0.21
(males, *n* = 27)	18.67–22.56	6.72–8.77	8.40–10.71	8.01–10.46	4.39–5.63	5.81–7.43	6.31–7.82	1.72–2.60
P. randi (*n* = 33)	17.11±0.68	6.53±0.30	8.09±0.33	7.8±0.32	4.19±0.29	5.68±0.27	5.84±0.33	1.75±0.14
Cerro Masvale	17.05 ± 0.69	8.28 ± 0.36	8.07 ± 0.35	7.81 ± 0.34	4.15 ± 0.28	5.66 ± 0.27	5.86 ± 0.34	1.72 ± 0.13
(males, *n* = 29)	15.80–18.65	7.53–9.27	7.38–8.73	7.09–8.34	3.50–4.62	5.09–6.40	5.21–6.79	1.39–2.00
Cerro Masvale	18.81 ± 0.66	6.83±0.32	8.19±0.17	8.44±0.12	4.52±0.25	5.87±0.37	5.90±0.2	1.91±0.16
(females, *n* = 4)	18.26–19.71	6.52–7.16	8.00–8.34	8.30–8.53	4.27–4.78	5.45–6.09	5.77–6.13	1.72–2.01
Puerto Inca	17.58±0.29	6.52±0.24	8.22±0.16	7.78±0.13	4.46±0.27	5.87±0.22	5.72±0.23	1.93±0.07
(males, *n* = 4)	17.26–17.95	6.26–6.80	8.05–8.37	7.59–7.87	4.18–4.76	5.69–6.20	5.50–5.96	1.83–2.00

Table 1.2. Call parameters of *Physalaemus randi*, *P. montubio*, and *P. pustulatus* from western Ecuador (ranges are in parenthesis). The calls of *P. montubio* and *P. randi* consist of one note with an amplitude-modulated portion ("first component") followed by a nearly pure tone with a frequency sweep ("second component"). Specimen's catalog no. at the Museo de Zoología de la Pontificia Universidad Católica del Ecuador (QCAZ) are shown. Unless otherwise indicated, samples sizes (number of calls) are: QCAZ 19752 = 17, QCAZ 23462 = 16, QCAZ 19517 = 13, QCAZ 19524 = 13, QCAZ 19518 = 10. Abbreviations are MFF = mean fundamental frequency, FC = first component, SC = second component. See text for details.

Specimen	Mean duration of each call (ms)	Call repetition rate (notes/min)	Mean interval between calls (ms)	Rise time (ms)	Mean No. of harmonics	MFF of the FC (KHz)	MFF of the SC (KHz)	Mean No. of pulses in FC
P. randi (QCAZ 19752)	241 (231–247)	114.3	284 (232–355)	68 (56–93)	4.0	1.314 (1.141–1.351; *n* = 9)	0.997 (0.985–1.006)	14.53 (14–15)
P. randi (QCAZ 23462)	246 (225–267)	120.5	252 (202–340)	75 (57–89)	5.1 (5–6)	1.358 (1.305–1.434)	0.925 (0.915–0.936)	13.00 (11–15)
P. montubio (QCAZ 19517)	82 (79–86)	218.2	193 (176–216)	14 (8–24)	5.5 (5–6)	1.369 (1.308–1.448; *n* = 5)	1.081 (1.071–1.087)	2.92 (2–5)
P. montubio (QCAZ 19524)	68 (63–76)	227.3	196 (138–314)	14 (10–29)	4.0	1.336 (1.114–1.550)	0.977 (0.931–1.001)	2.40 (2–3)
P. pustulatus	475	17.4	2995	130	5.6	--	0.723	--

Specimen	Mean duration of each call (ms)	Call repetition rate (notes/min)	Mean interval between calls (ms)	Rise time (ms)	Mean No. of harmonics	MFF of the FC (KHz)	MFF of the SC (KHz)	Mean No. of pulses in FC
(QCAZ 19518)	(397–535)		(1629–6553)	(114–139)	(5–6)		(0.650–0.744)	

Table 1.3. Character loading and percentage of explained variance for Principal Components (PC) I–III for eight morphometric variables. To remove the effect of "size," linear regressions were performed between all variables and snout-vent length (SVL). The PC analysis was applied to SVL and the residuals from the regressions with the other variables.

Variable	Size-free morphology		
	PC I	PC II	PC III
SVL	< 0.00001	< 0.00001	1.000
Residual dorsum width	0.545	–0.604	< 0.00001
Residual tibia length	0.778	0.177	< 0.00001
Residual femur length	0.706	–0.137	< 0.00001
Residual arm length	0.426	0.607	< 0.00001
Residual head length	0.603	0.304	< 0.00001
Residual head width	0.579	–0.516	< 0.00001
Residual eye-nostril distance	0.299	0.486	< 0.00001
Eigenvalue	2.37	1.378	1.000
%	39.6	17.2	12.5

Figure 1.1. Dorsolateral view of the holotype of *Physalaemus randi*, QCAZ 19563 (adult male from Cerro Masvale, Ecuador).

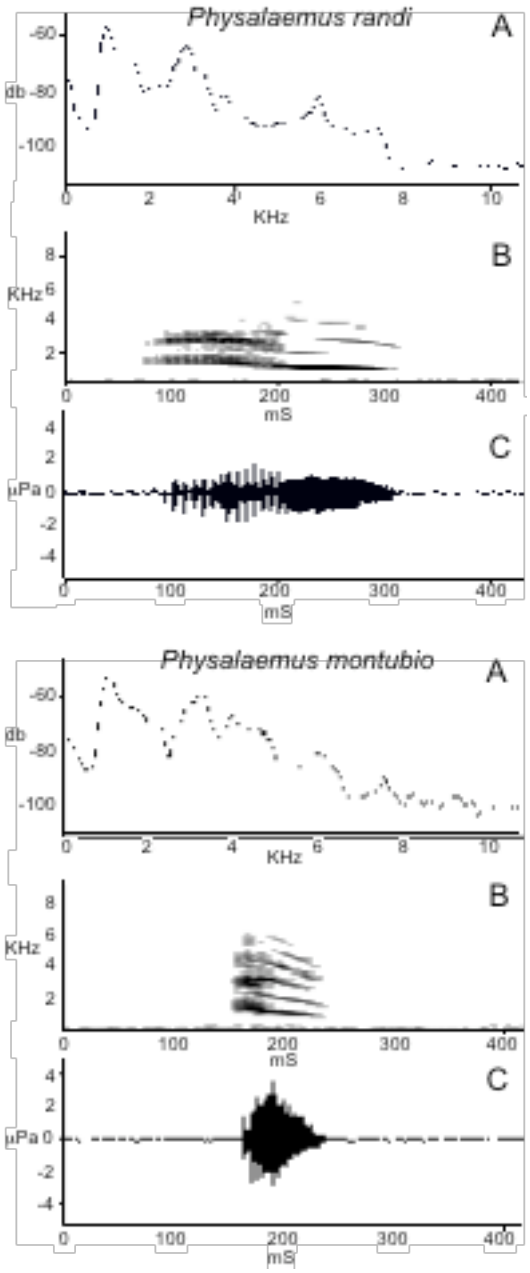

Figure 1.2. (A) Power spectrum, (B) sonogram, and (C) oscillogram of the advertisement call of *Physalaemus randi* (QCAZ 19752), and *P. montubio* (QCAZ 19517). The power spectra were measured along the entire duration of the calls.

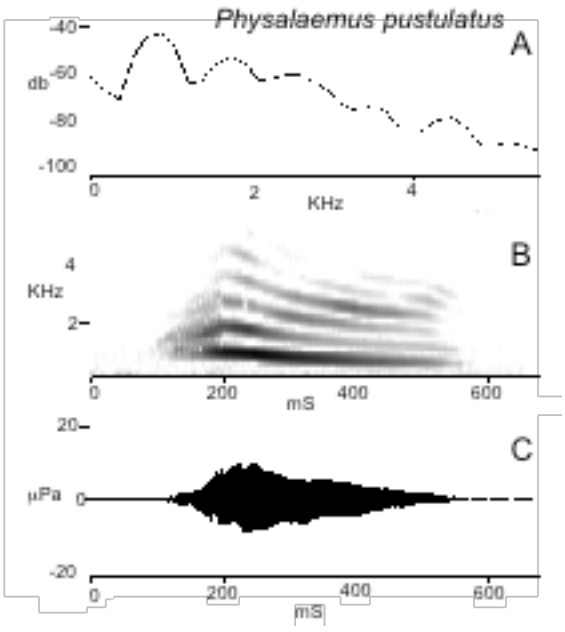

Figure 1.3. (A) Power spectrum, (B) sonogram, and (C) oscillogram of the advertisement call of *Physalaemus pustulatus* (QCAZ 19518). The power spectrum was measured along the entire duration of the call.

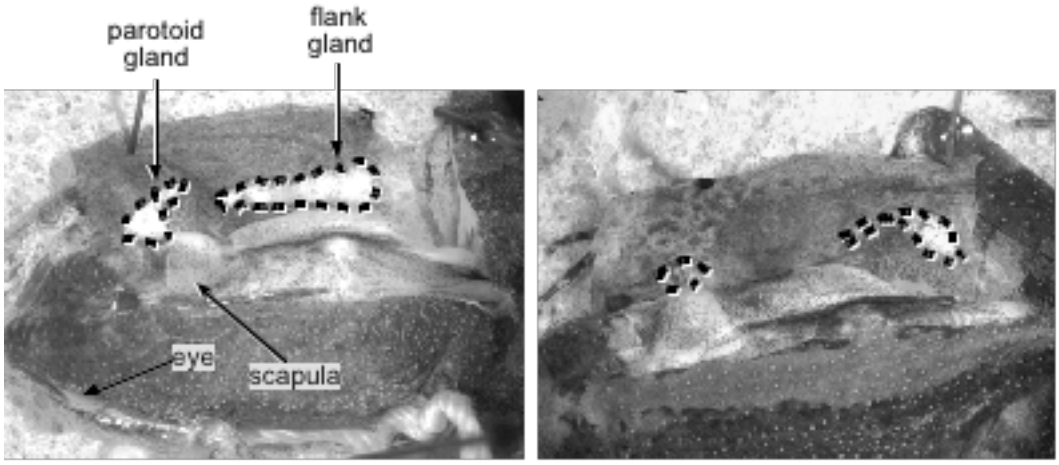

Figure 1.4. Dorsal view of dissected specimens showing flank and parotoid glands. The skin has been uplifted and its internal surface is showing. Left: *Physalaemus randi*, QCAZ 19579, SVL = 17.98 mm; right: *P. montubio,* QCAZ 19530, SVL = 21.96.

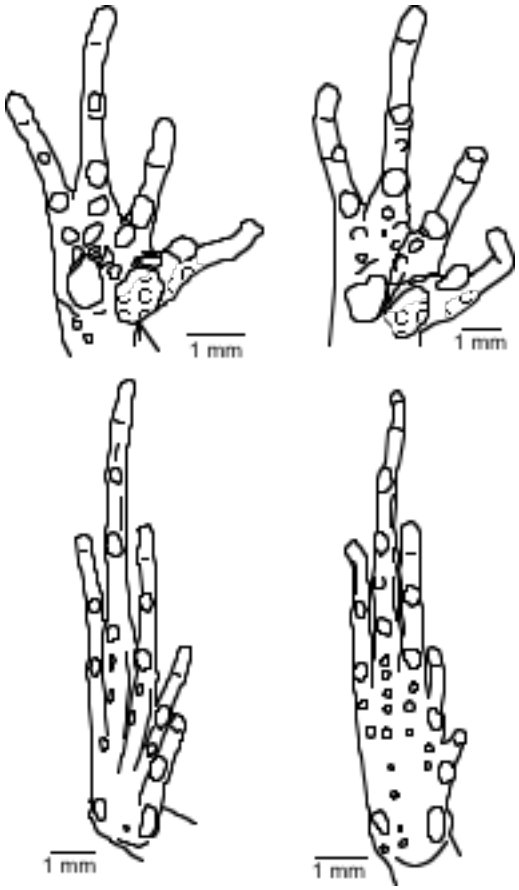

Figure 1.5. Ventral views of the hand and foot of the holotype of *Physalaemus randi* (left), QCAZ 19563 (adult male from Cerro Masvale, Ecuador) and holotype of *P. montubio* (right), QCAZ 19524 (adult male from Puerto Rico, Ecuador).

Figure 1.6. Dorsal views of adult *Physalaemus randi* showing variation in dorsal patterns. Left to right: QCAZ 19584 (female), QCAZ 19575 (male), QCAZ 19563 (male, holotype), QCAZ 19580 (male). All from Cerro Masvale (Provincia Manabí, Ecuador).

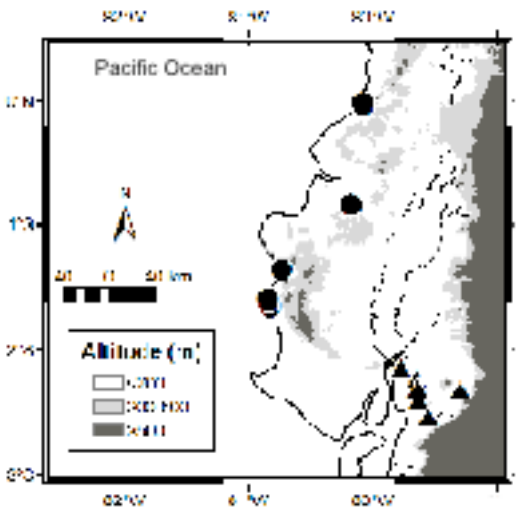

Figure 1.7. Known records of *Physalaemus randi* (triangles) and *P. montubio* (circles).

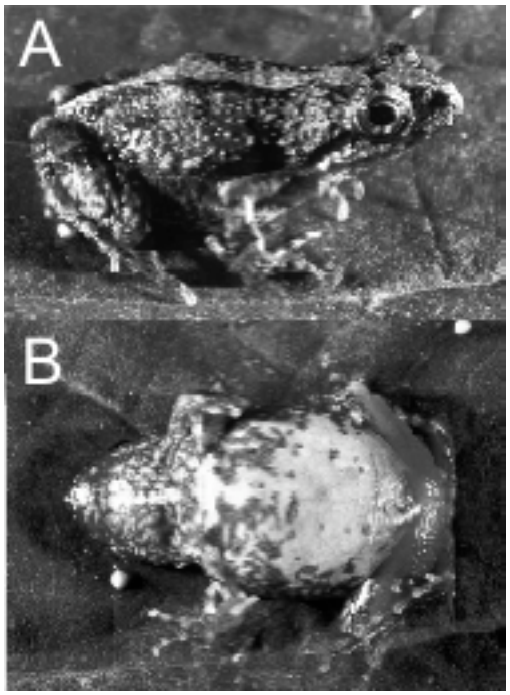

Figure 1.8. Dorsolateral (A) and ventral (B) views of *Physalaemus montubio*, QCAZ 19512 (adult female from Puerto Rico, Ecuador).

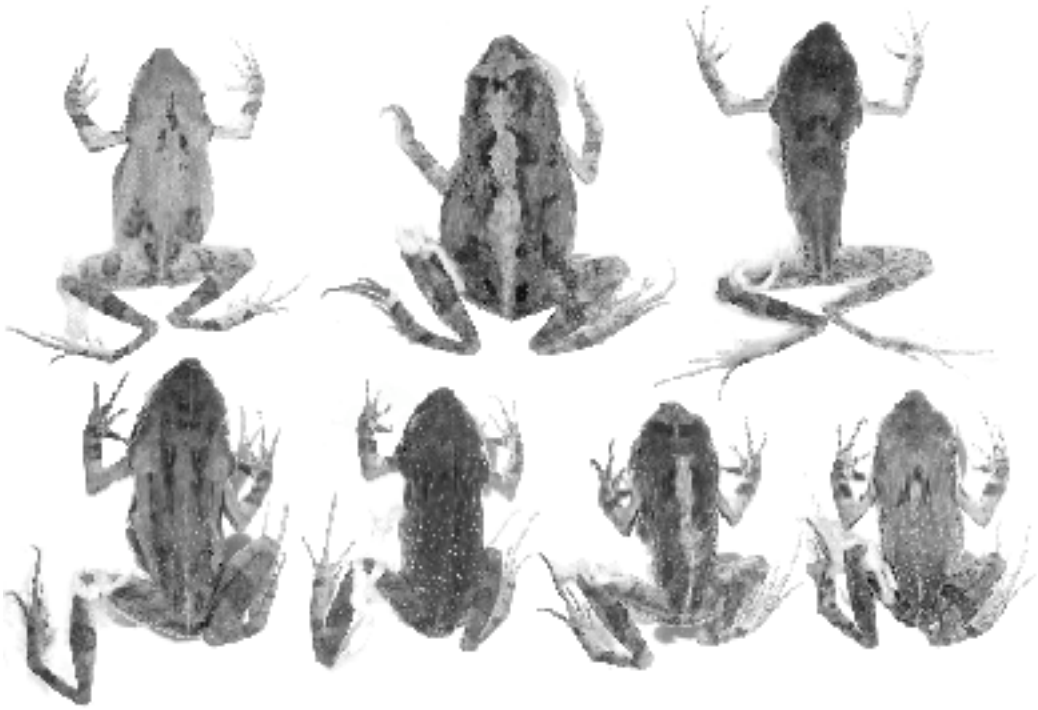

Figure 1.9. Dorsal views of adult *Physalaemus montubio* showing variation in dorsal patterns. Upper row (left to right): QCAZ 12341 (male, Río Chico), QCAZ 19512 (female, Puerto Rico), QCAZ 19520 (male, Puerto Rico). Lower row (all males, Puerto Rico): QCAZ 19524, QCAZ 19530, QCAZ 19552, QCAZ 19555. All from Provincia de Manabí, Ecuador.

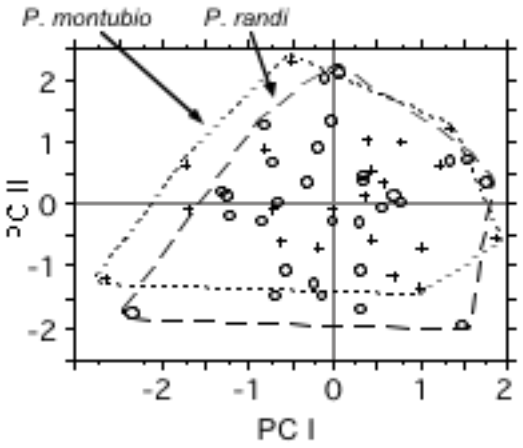

Figure 1.10. Axes I and II from Principal Components Analysis based on snout-vent length and seven size-corrected morphological variables for 21 specimens of *Physalaemus montubio* from Puerto Rico (+), and 30 of *P. randi* from Cerro Masvale and Puerto Inca (O).

Supplemental Data 1.1. Examined Specimens

Physalaemus coloradorum: Ecuador: Pichincha: 1 km NW from La Florida, 1003 m, QCAZ 19373–74, 19439–41

Physalaemus petersi: Bolivia: Cochabamba: 6.5 km N Chipiriri, 260 m, KU 135513–16. Ecuador: Orellana: Estación Científica de la Universidad Católica del Ecuador, Parque Nacional Yasuní, 240 m, QCAZ 14733–38

Physalaemus pustulatus: Ecuador: Provincia de Manabí: Puerto Rico, 10 m, QCAZ 19355, 19513–14, 19518, 19523, 19537, 19541–42, 19545–48, 19551, 19553–54. Provincia de Los Ríos: Patricia Pilar, 200 m, QCAZ 19538–40, 19605–14, 19745–48. Provincia del Guayas: Guayaquil, MCZ 7666 (holotype). Provincia del Guayas: Isla Puná, CAS 5408. Provincia del Guayas: Cerro Blanco, QCAZ 23427.

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Chapter 2

A New, Cryptic Species of *Physalaemus* (Anura: Leptodactylidae) from Western Ecuador with Comments on the Call Structure of the *P. pustulosus* Species Group*

Abstract. I describe a new species of leptodactylid frog of the genus *Physalaemus* from the lowlands of western Ecuador. It belongs to the *P. pustulosus* species group and differs from other group members in its smaller size, skin texture, tadpole characters, and advertisement call. The new species has wide variation in size and color pattern both at the intra- and inter-population levels. This variation matches that observed in *P. montubio* and *P. randi*, and render difficult its diagnosis on the basis of morphological features. A phylogenetic analysis of morphological characters shows that the new species belongs to a clade distributed west of the Andes in Ecuador and northern Peru, sister to (*P. petersi* + *P. pustulosus*). A phylogeny based on mtDNA show that *P. montubio* and *P. randi* form a clade sister to (*P. coloradorum* + *P.* sp. nov.). The calls of these three species have two components with different acoustic features that arguably match the frequency sensitivity of the ear of conspecific females, in a manner similar to the complex calls of *P. pustulosus*. The new species occurs in two predominantly dry regions, the Lowland Deciduous Costa Forest, and the Lowland Semideciduous Costa Forest.

*Significant portions of this chapter have been previously published as Ron, Coloma, & Cannatella, 2005. Herpetologica 61:178–198. Throughout this chapter, for consistency with the original publication, the species of the *pustulosus* group are assigned to the genus *Physalaemus*. Nascimento et al. (2005) subsequently assigned these species to the genus *Engystomops* and this nomenclatural change is fallowed in Chapters 3, 4, and 5.

2.1 INTRODUCTION

Ecuador has one of the highest amphibian species richness, with a total of 429 described species (updated from Coloma and Quiguango, 2000–2003). Within Ecuador, the biogeographic regions with the highest regional diversities are the montane forests of the Andean slopes. Conversely, the most depauperate amphibian communities are those from the driest habitats in Ecuador, the Dry Costa Shrub and the Deciduous and Semideciduous Costa forest (Ron, unpublished; vegetation types as defined in Sierra et al., 1999).

Although covering an area of approximately 33,700 km^2 (Sierra et al., 1999) only 25 amphibian species have been recorded in these dry habitats. At least 19 of those amphibians have significant portions of their distribution ranges in adjacent habitat types, especially the Chocoan Tropical Rainforest. The remaining species have distributions restricted (or nearly so) to dry habitats and are therefore unique elements of the amphibian fauna. These species are *Ceratophrys stolzmanni, Leptodactylus labrosus, Physalaemus pustulatus, Colostethus infraguttatus, C. elachyhistus,* and *Rana bwana*.

Surveys carried out in 2002 and 2003 at the Deciduous and Semideciduous Costa forest have resulted in additions to this short list. Two species of *Physalaemus* belonging to the *P. pustulosus* species group have been described recently (Ron et al., 2004). Herein, I describe an additional species of the same group from the lowlands of western Ecuador. During fieldwork in 2002, specimens of the new species initially were misidentified as *P. montubio* because of similarities of their calls and external morphology. However, analyses of mitochondrial DNA and a careful examination of the morphology of adults made evident the distinctiveness of this new species from other members of the *P. pustulosus* group.

2.2 MATERIALS AND METHODS

Morphological terminology and abbreviations follow Lynch and Duellman (1997) for adults and Altig and McDiarmid (1999) for tadpoles. Osteological characters used in the diagnosis were examined in clear-and-stained (C & S) specimens and are defined in Cannatella and Duellman (1984) and Cannatella et al. (1998). Sex was determined by the presence of nuptial pads, vocal sac folds, and/or by gonadal inspection. Tadpoles were staged according to Gosner (1960). Snout-vent length is abbreviated as SVL throughout. Examined specimens (listed in the type-series and Supplemental Data 2.1) are housed in the American Museum of Natural History (AMNH), California Academy of sciences (CAS), Museo de Zoología de la Pontificia Universidad Católica del Ecuador (QCAZ), Museum of Comparative Zoology Harvard University (MCZ), National Museum of Natural History (NMNH), and Natural History Museum University of Kansas (KU).

Geographic coordinates for specimens collected in 2002 and 2003 were measured with a Garmin™ 12CX GPS receiver, based on the geodetic datum WGS 84.

Sound recordings were made with a Sennheiser™ ME-67 directional microphone and a Sony™ WM-D6C analog tape recorder. Calls were analyzed using Canary™ 1.2.1 software (Charif et al., 1995) at a sampling frequency of 22.1 kHz and a frequency grid resolution (FTP) = 8192. I measured eight call parameters: (1) call duration (time from beginning to end of call); (2) rise time (time from the beginning of the call to the point of maximum amplitude); (3) interval between calls (time between the end of one call and the beginning of the next call); (4) duration of the first component (time from beginning of the first pulse to the end of the last pulse of the first component of the call, expressed as percentage of call length); (5) call repetition rate (number of calls/min); (6) fundamental frequency of the first component (frequency of the first harmonic along the duration of the first component of the call); (7) dominant frequency of the first component (frequency with the most energy along the duration of the first component of the call); and (8) fundamental frequency of the second component (frequency of the first harmonic along the duration of the second component of the call). Original recordings are deposited in the audio archive of the QCAZ and the Texas Natural History Collection of the Texas Memorial Museum (University of Texas at Austin).

Shape and size of parotoid glands and flank glands were determined by making a middorsal incision to reflect the skin, which allowed for inspection of its internal surface. Length of the parotoid gland and flank gland were measured from the anterior to the posterior edge. To avoid specimen deterioration, I measured gland length of only one

third of the available specimens. All well-preserved specimens (Simmons, 2002) were measured for the following morphometric variables: (1) snout-vent length; (2) head length; (3) head width; (4) eye-nostril distance; (5) femur length; (6) tibia length; (7) arm length; and (8) dorsum width. Measurements were made according to the methodology described in Duellman (1970) and Ron et al. (2004) with Fowler digital calipers (nearest 0.01 mm) from specimens fixed in 10% formalin and preserved in 70% ethanol. Because of small sample sizes for females, most morphometric comparisons were made only among adult males. Tadpole measurements were taken from digital images using the measurement tool of Adobe® Photoshop® 7.0 (Adobe Systems Incorporated, 2002).

Multivariate analyses were used to assess the degree of morphometric differentiation among species. To remove the effect of covariation with SVL, Principal Components Analysis (PCA) and Discriminant Function Analysis (DFA) were applied to the residuals of the linear regressions between the seven measured variables and SVL (Vitt et al., 2000). For the PCA, only components with eigenvalues > 1 were retained. The species included in the analyses are (number of specimens in parentheses): *P.* sp. nov. (55); *P. montubio* (101); *P. pustulatus* (24); and *P. randi* (35). StatView® 5.01 (SAS Institute Inc., 1998) was used to perform the PCA; JMP® 4.0 (SAS Institute Inc., 2000) was used to perform the DFA.

Climate parameters for localities were estimated from digital climate maps compiled by C. Graham, using ArcMap 8.2 with the SpatialAnalyst extension (ESRI, 2001).

Phylogenetic Analyses based on morphological characters. Phylogenetic analyses were based on morphological and mtDNA sequences. I expanded the morphology-based phylogenetic analysis made by Cannatella et al. (1998) to include the recently described species (herein and *P. montubio* from Ron et al., 2004). In the Cannatella et al. (1998) phylogeny, *Physalaemus randi* and *P. pustulatu*s are mistakenly referred as "*P. pustulatus*" and "P. sp. C", respectively). States of characters 1–12 were scored following Cannatella et al. (1998), except that the flank gland was scored as absent or present. The only addition to the matrix was character 13 (SVL); it was scored by step-matrix gap-weighting (scaled to 999, Supplemental Data 2.2; Wiens, 2001). Between-character scaling was used to give the same maximum length to binary characters (qualitative; 1–12) and quantitative characters (SVL; Wiens, 2001); non-step-matrix characters have a weight of 999. *Physalaemus enesefae* and *P. ephippifer* were used as outgroups. Parsimony analyses were performed with PAUP* 4.08 (Swofford, 2000) using the exhaustive search algorithm. The data matrix is shown in Supplemental Data 2.2.

Phylogenetic Analyses based on molecular characters..—We sampled 11 populations of *Physalaemus* from western Ecuador, belonging to five species. The primary purpose the molecular analyses presented here is to provide evidence of the distinctiveness of *P. guayaco* with respect to its more closely related species. A more comprehensive phylogeny including all species of the species group will be published elsewhere. DNA was extracted from liver tissue samples stored in 95% ethanol. I analyzed 2409 bases of mitochondrial DNA genes12S rRNA, valine-tRNA gene, and 16S rRNA. Methods for DNA extraction, amplification, and sequencing followed the protocol of Santos et al.

(2003). Preliminary alignment was done with CLUSTAL X 1.8 (Thompson et al., 1997) over a matrix containing *P. pustulosus*, *P. freibergi*, *P. petersi*, and 25 species of *Physalaemus* outside the *P. pustulosus* species group. The ambiguously-aligned regions were adjusted by eye to produce a parsimonious alignment (i.e., informative sites minimized). Parsimony and maximum likelihood (ML) analyses were performed with PAUP* 4.08 (Swofford, 2000). The most parsimonious tree was found with a branch-and-bound search and the ML tree with heuristic searches using TBR branch swapping, random addition sequence of taxa and 10 replicates per search. Characters were unordered and equally weighted for parsimony analyses. Maximum likelihood searches were performed under six nested models of nucleotide substitution. The best fitting model was GTR + Γ according to a the ML ratio test (Huelsenbeck and Crandall, 1997). Parsimony and ML analyses (all models) yielded trees with identical branching pattern. *Physalaemus pustulatus* was used as an outgroup based on its position in the Cannatella et al. (1998) "combined" phylogeny (referred to as "*Physalaemus* sp. C" in that work). The basal position of *P. pustulatus* within the clade of *Physalaemus* distributed in northwestern South America is corroborated by the morphology-based phylogeny (Fig. 2.11) and additional unpublished mtDNA sequence data by DCC, including *P. pustulosus*, *P. freibergi*, *P. petersi*, and 25 species of *Physalaemus* outside the *P. pustulosus* species group. Locality of specimens and GenBank accession numbers are shown in Supplemental Data 2.3. In both the morphology and DNA-based phylogenies, clade support was estimated from non-parametric bootstrapping with heuristic searches of 1000 replicate data sets.

2.3. RESULTS

Physalaemus ***guayaco*** sp. nov

Holotype. (Fig. 2.1) QCAZ 23521 (field no. PUCE 8430), adult male from Ecuador, Provincia del Guayas, Cerro Masvale (private reserve at 2.394° S, 79.642° W), 92 m, collected by M. R. Bustamante, I. G. Tapia, and S. R. Ron on 23 March 2003.

Paratopotypes. QCAZ 19381–82, 19561–62, 19751, adult males, collected by D. C. Cannatella, L. A. Coloma, A. Holloway, and S. R. Ron on 20 February 2002; QCAZ 23505–12, 23514–15, 23516 (C & S), 23517, 23519–21, adult males, 23518 (C & S), adult female, collected by M. R. Bustamante, I. G. Tapia, and S. R. Ron on 23 March 2003.

Paratypes. Ecuador: Provincia del Guayas: 20 km E of Durán on the road to Milagro (2.022° S, 79.690° W), 32 m, QCAZ 23445, adult male; 11 km N Cerro Masvale, on the road to Virgen de Fátima (2.300° S, 79.639° W), 40 m, QCAZ 23522, 23525–27, 23532–37, 23541, 23559–570, 23571 (C & S), 23572, 23573 (C & S), 23574, 23575 (C & S), 23576–78, adult males, 23531, adult female; 15 km S Naranjal, on road to Machala (2.766° S, 79.692° W), 74 m, QCAZ 23650, 23652–53, 23655–56, adult males, 23654, adult female, 23651 juvenile. Collected by M. R. Bustamante, I. G. Tapia, and S. R. Ron between 20 and 25 March 2003.

Diagnosis. A member of the *P. pustulosus* group, sensu Cannatella and Duellman (1984) and Cannatella et al. (1998). The assignment to the *P. pustulosus* group is based on the presence of four synapomorphies (Cannatella et al., 1998): (1) presence of flank glands;

(2) presence of parotoid glands; (3) warty skin; and (4) dentigerous process of the vomer thin and spikelike.

Physalaemus guayaco (Fig. 2.1) is characterized by: (1) mean SVL 16.85 mm in males (range 15.45–19.38; *n* = 55) 18.67 mm in females (range 16.77–20.98; *n* = 3); (2) skin on dorsum bearing scattered tubercles; (3) snout varying between truncate and subacuminate in dorsal view and round in lateral view; (4) vomerine teeth and odontophores absent; (5) maxillary and premaxillary teeth present; (6) parotoid glands present, mean length = 1.90 mm (*n* = 23; SD = 0.50; 6.3–15.6% of SVL); (7) flank glands present, mean length = 3.93 mm (*n* = 23; SD = 1.14; 10.3–32.1% of SVL); (8) tarsal tubercle absent; (9) nuptial pads present; (10) Finger I shorter than II; (11) tympanic annulus evident, concealed dorsally, tympanic membrane not tuberculate.

Physalaemus guayaco is smaller than *P. petersi, P. pustulosus,* and *P. pustulatus* (non overlapping SVL; Cannatella and Duellman, 1984; Ron et al., 2004) and has a different advertisement call (Ron et al., 2004; Ryan and Rand, 2001; Fig. 2.2). The absence of a tarsal tubercle and the presence of teeth in the maxilla and premaxilla further distinguish it from *P. petersi* and *P. pustulosus.* The tadpole of *P. guayaco* differs from that of *P. petersi* in lacking paired elliptical paravertebral glands and in dorsal coloration (Fig. 2.3). *Physalaemus coloradorum* has a longer advertisement call without well-defined pulses at the beginning (Ryan and Rand, 2001), and bigger dorsal tubercles, some coalescing into ridges (tubercles are more scattered and never coalesce into ridges in *P. guayaco*; Fig. 2.4). *Physalaemus coloradorum* further differs from *P. guayaco* in having a vertical loreal region (oblique in *P. guayaco*). *Physalaemus guayaco* is most similar to

P. montubio and *P. randi*; the feet of both species have less extensive lateral fringes and basal webbing than *P. guayaco*. *Physalaemus guayaco* has a shorter call (Fig. 2.2; Table 2.1; Ron et al., 2004) and smaller and less abundant dorsal tubercles than *P. randi* (Fig. 2.4). *Physalaemus montubio* is larger than *P. guayaco* (male mean SVL = 20.6 mm [n = 117; SD = 1.16; specimens from eight populations] and 16.85 [n = 55; SD = 0.99; from three populations], respectively; t = 20.66; df = 170; $P < 0.0001$; Fig. 2.5). Size differences are not significant between the population of *P. montubio* with the lowest average SVL (52 km W El Carmen on the road to Pedernales, Provincia de Manabí) and that of *P. guayaco* with the highest average SVL (15 km S Naranjal, Provincia del Guayas; t = 0.79; df = 8; P = 0.45). However, these populations are 630 km apart, at the opposite latitudinal extremes of their species distribution ranges, and therefore are unlikely to represent part of a size gradient within a single species (Figs. 2.5, 2.6). Calls of *P. montubio* resemble those of *P. guayaco* except for a small difference in frequency: average fundamental frequency for the first component of the call = 1.475 kHz in *P. montubio* (SD = 104.8; 21 males, 7 populations) and 1.576 kHz in *P. guayaco* (SD = 0.153; Table 2.1). Differences are close to significant (t = 1.782; df = 24; P = 0.087), although they may be explained by significant differences in SVL between both samples (*P. montubio* mean SVL = 20.06 mm [SD = 1.35]; *P. guayaco* = mean SVL 17.67 mm [SD = 1.53]; t = 3.47; df = 24; P = 0.002). Phylogenetic analysis of morphological characters and 2374 bases of 12S and 16S rRNA genes of mtDNA show unambiguously that, despite the similarity of their advertisement calls and external morphology, *P. guayaco* and *P. montubio* are not sister species (see Discussion).

Description of holotype.—Adult male, 16.02 mm SVL, tibia length 7.55 mm, femur length 7.55 mm, arm length 3.69 mm, head length 5.61 mm, head width 5.27 mm, eye-nostril distance 1.81 mm, dorsum width 5.91, head wider than body except in scapular region; diameter of eye twice diameter of tympanic annulus; tympanic membrane and tympanic annulus barely evident, hidden dorsally; tympanic annulus ovoid, longer dorsoventrally; tubercles absent from tympanic membrane or annulus; supratympanic fold absent; head slightly elevated between orbits and flat in intercanthal region; snout rounded in profile and truncated in dorsal view; nostrils slightly elevated, internarial region concave; canthus rostralis rounded; loreal region convex with shallow groove extending from posterior border of nostril to posteroventral border of orbit**.**

Fingers without expanded discs; nuptial pad present, keratinized, brown, divided in two portions, one covering posterior half of thenar tubercle, other covering base of Finger I. Base of palmar and thenar tubercles ovoid; palmar tubercle less prominent than thenar tubercle; subarticular tubercles with round base, all conical except for round and low second (distal) subarticular tubercle on Finger III; second subarticular tubercle on Finger IV absent; few supernumerary palmar tubercles present. Webbing between fingers absent; relative lengths of adpressed fingers III > IV > II > I. Toes without expanded discs; base of inner metatarsal tubercle ovoid, larger than ovoid base of outer metatarsal tubercle; inner metatarsal tubercle more prominent than outer metatarsal tubercle; subarticular tubercles with round base, all conical except for subconical distal subarticular tubercles of Finger IV and V; sparse and minute conical plantar supernumerary tubercles; tarsal tubercle absent; lateral fringes on toes; fringes converge

at the base of adjacent toes forming basal webbing; relative lengths of adpressed toes IV > III > V > II > I.

Skin on dorsum bearing minute, round to subconical tubercles, widely scattered anteriorly, some arranged in longitudinal rows; skin on venter smooth. Tongue longer than wide; vomerine teeth and odontophores absent. Maxillary and premaxillary teeth present. Vocal slits present, parallel to margins of mandible. Deflated vocal sac forming folds on gular region, extending posteriorly to proximal end of arm.

Color of holotype in preservative. Dorsum grayish brown with darker hue on the head, particularly above and between orbits and anterior half of snout; dark gray longitudinal bands weakly defined, running on each side of dorsum, parallel to middorsal axis from scapular to sacral region; faint light gray middorsal line from snout tip to vent, interrupted on intercanthal and interscapular regions; dorsal tubercles light gray; dorsal surfaces of forearms and hind limbs light gray with dark gray marks on forearms and elbows. Venter cream yellowish without marks along posterior one-third of SVL; scattered dark-gray minute spots on chest, some aggregated on dark blotches; minute dark gray spots abundant on ventral surfaces of head except for weakly defined irregular immaculate blotches along jaw margins; ventral surfaces of hind limbs and arms cream yellowish, becoming light gray towards outer and inner edges, some dark blotches present; outer half of ventral surfaces of forearms dark gray; sides of head gray with a light gray area below the orbit and tympanum, extending posteriorly above deflated vocal folds; flanks dark gray dorsally, light gray ventrally; gray appearance on ventral and

lateral surfaces of head, limbs, and flanks, results from composite effect of numerous minute melanophores.

Color in life. (QCAZ 23506, adult male) dorsum grayish-brown with dark marks; subocular bar cream; flanks gray; venter reddish-cream posteriorly; anterior half of venter cream with gray blotches; ventral surfaces of head dark gray with cream midventral stripe from jaw tip to scapular region; thighs salmon red ventrally; iris bronze (S. Ron, field notes).

Etymology. The specific name *guayaco* is a noun in apposition, in reference to the inhabitants of Guayaquil and Provincia del Guayas, Ecuador.

Variation.—Variation in the dorsal coloration of preserved specimens is extensive (Fig. 2.7). The background dorsal coloration varies from light gray (QCAZ 23655) to dark gray (QCAZ 23527, 23532). Irregular dark marks may be present in diverse patterns (Fig. 2.7). The mid-dorsal line varies between well-defined and continuous (from snout tip to vent; QCAZ 23531, 23533, 23652, 23654; Fig. 2.7), to weakly-defined, restricted to the posterior one-third of the sacral region (QCAZ 23653, 23574; Fig. 2.7), or absent (QCAZ 23537, 23534; Fig. 2.7); the line is enclosed within a longitudinal light gray band of irregular width in QCAZ 23656, and 23654 (Fig. 2.7). In 31 specimens from the type series (e.g., QCAZ 23534, 23561, 23574, 23576; Fig. 2.7) there is a lighter area in the scapular region. There is also variation in the abundance and arrangement of tubercles (all lighter than the background).

The ventral surfaces of all preserved specimens have a cream to yellowish-cream background with light gray (QCAZ 23564, 23568) to dark gray markings (QCAZ 23653,

23531). Marks may be restricted to the head (darker on folded vocal sacs; QCAZ 23650, 23653) or also present over the entire venter (less abundant posteriorly, QCAZ 23566). Variation between the extremes is continuous. In a few specimens, the ventral marks are arranged in well-defined large spots (QCAZ 23512, 23655). More frequently, these marks form diffuse speckled patterns (e.g., QCAZ 23525, 23560). A midventral cream stripe, extending from near the tip of the snout to the chest, is present in QCAZ 23537. The stripe can be restricted to the gular region (QCAZ 23522) or absent (QCAZ 23569). The arrangement of dark spots and tubercles on the ventral surfaces of the feet and hands of QCAZ 23515 is shown in Figure 2.8.

Head shape varies continuously from truncate (QCAZ 23563) to subacuminate (QCAZ 23564, 23566). Lateral head coloration varies between light gray and dark gray. The light area below the eye and tympanum is cream in most specimens (e.g., QCAZ 23567). It may be interrupted by a dark bar below the orbit (QCAZ 23563, 23576) or restricted to a thin longitudinal stripe bordering the orbit and tympanum (QCAZ 23655). There is a dark-gray labial bar below the loreal region in QCAZ 23564 and 23652.

The following morphometric data pertain only to adults. In the type series, the largest male has a SVL of 19.38 mm, and the largest female 20.98 mm; mean male SVL = 16.85 mm (n = 55; SD = 0.99), mean female SVL = 18.67 mm (n = 3; SD = 2.13). The small sample size of females prevents analysis of size dimorphism; however, the available data suggest that females are larger than males. The only female from 11 km N Cerro Masvale (SVL = 18.26 mm; QCAZ 23531) is larger than 96.7% of the males of the same locality (n = 30); the only female from 15 km S Naranjal (SVL = 20.98; QCAZ

23654) is larger than all the males (n = 5); the only female from Cerro Masvale (SVL = 16.77; QCAZ 23518) is larger than 40% of the males (n = 20).

Snout-vent length is not significantly different between males of Cerro Masvale and those from 11 km N Cerro Masvale (t = 1.80; df = 48; P = 0.08), or from Cerro Masvale and those from 15 km S Naranjal (t = 4.48; df = 23; P = 0.15). Snout-vent length was significantly different between specimens from 11 km N Cerro Masvale and those from 15 km S Naranjal (t = 3.01; df = 33, P = 0.005). Descriptive statistics of morphometric measurements for the three populations are given in Table 2.2.

All morphometric variables shown in Table 2.2 show a significant positive relation with SVL (simple regressions for males; all ANOVA's $P < 0.005$; df = 52). The relation also is significant between SVL and flank gland length (F = 5.71; df = 21; P = 0.03) and between SVL and parotoid gland length (F = 6.64; df = 21; P = 0.02; specimens from Cerro Masvale and 11 km N Cerro Masvale combined). Flank gland length is correlated with parotoid-flank length (F = 15.76; df = 21; $P < 0.001$). Total gland length (flank + parotoid) varies between 16.6% of SVL (QCAZ 23567 from 11 km N Cerro Masvale) and 45.6% of SVL (QCAZ 23516 from Cerro Masvale). In all specimens, parotoid and flank glands are separated from each other.

Specimens from 11 km N Cerro Masvale have proportionally shorter flank and parotoid glands than those from Cerro Masvale (Mann-Whitney's U for flank gland length/SVL = 2; $P < 0.0001$; U for parotoid gland length/SVL = 21; P = 0.010; 10 specimens from 11 km N Cerro Masvale and 12 from Cerro Masvale, all males).

Three morphometric variables show proportional differences among populations: (1) dorsum width (Kruskal-Wallis *H* for dorsum width/SVL = 10.84; *P* = 0.004); (2) arm length (Kruskal-Wallis *H* for arm length/SVL = 8.80; *P* = 0.01); and (3) head width (Kruskal-Wallis *H* for head width/SVL = 7.58; *P* = 0.02). Other variables listed in Table 2.2 do not show significant statistical differences (all Kruskal-Wallis, *P* > 0.09).

Tadpoles. The following description is based on a lot (QCAZ 24006A–D) of four larvae in Stages 28 (A), 29 (B–C), and 31 (D). Tadpoles were raised in laboratory conditions from a clutch of eggs, that were laid in captivity by female QCAZ 23654, collected on 15 km S Naranjal (Provincia del Guayas) by S. R. Ron, M. R. Bustamante, and I. Tapia on 23 March 2003 at 2100 h. Tadpoles were preserved in formalin 10% on 25 March, 30 April, and 6 May 2003.

These larvae belong to the exotrophic, lentic, bentic ecomomorphological guild as defined by McDiarmid and Altig (1999). Morphometric data are provided in Table 2.3. A Stage 28 tadpole shows, in dorsal view (Fig. 2.3), an elliptical body, widest between eye and spiracle, with a rounded snout. Eyes are relatively large (BL about 7.5 times larger than ED), separated by a distance equal to about 1.9 times the internarial distance, directed and positioned dorsolaterally, not visible in ventral view. External nares oval, located dorsolaterally, at about one third the distance between anterior margin of snout and anterior margin of eye.

In profile (Fig. 2.3) body depressed (BW/BH = 1.4), flattened ventrally, snout rounded. Oral disc posteriorly emarginated. Spiracle sinistral, cylindrical inside the body wall, but protruding laterally, its tip closer to the vent than to the eye, positioned

ventrolaterally and oriented posterodorsally. Spiracle opening rounded, situated at level of the hind limbs.

Tail musculature conspicuous, decreasing in size towards the tip of tail. Tail dorsal fin not extending onto body, slightly convex, and reaching its higher size at about midtail. Tail tip nearly rounded. Ventral fin almost straight, beginning just posterior to the vent, about equal size along its length. Anal tube dextral, tubular, linked to caudal muscle. Limb buds of about equal length than diameter. No lateral line or glands.

Oral disc ventral (Fig. 2.3), transversely elliptical; anterior labium bearing marginal papillae interrupted by a medial, dorsal gap extending two-fourths of the anterior margin of labium; posterior labium emarginated, bordered by two rows of small rounded papillae. LTRF 1/3; A2 = 7 + 3 labial teeth, interrupted by a medial gap; P1 = 14 + 17 labial teeth, interrupted by a right gap; P2 = 5 labial teeth; and P3 = 32 labial teeth. Jaw sheaths thin, serrated at inner side, upper nearly straight, lower angular and shorter than anterior.

In preservative, dorsum, flanks and caudal muscle pigmented by scattered melanophores arranged in a pattern depicted on Figure 2.3. Unpigmented areas include a small round spot between eyes at level of anterior margin of eyes, irregular areas around eyes, nares, spiracle, vent tube, fins, distal tail muscles, oral disc and venter. Tail musculature pigmented with scattered melanophores mostly on the anterior half. Tail fins transparent except on tip of tail, at which melanophores are denser than in adjacent areas forming a discrete, conspicuous mark. Venter of body nearly transparent but with

scattered melanophores forming a transverse, diffuse stripe that crosses the body at level of anterior branquial baskets. Color in life unknown.

Tadpole in Stage 29 (QCAZ 24006B–C) and Stage 31 (QCAZ 24006D) are similar in shape and color pattern to the one described previously. Differences are noted on the LRTF, the amount of labial teeth and its distribution. QCAZ 24006B and 24006D have a LRTF 2/2, whereas QCAZ 24006C has 2/3. In QCAZ 24006B, the number of labial teeth and its distribution is A1 =14, A2 = 17 + 18, P1 = 23, P2 = 28, whereas in QCAZ 24006C is A1 = 3 + 21 + 3, A2 = 16 + 20, P1 = 21 + 24, P2 = 39, P3 = 5, and in QCAZ 24006D is A1 = 49; A2 = 12 + 14; P1 = 44, P2 = 36. A2 and P1 bear larger teeth than the outer rows. A brown tail tip mark is present in all of them and is more conspicuous in C.

Among the species in the *P. pustulosus* group, only the larvae of *P. coloradorum* (Cannatella and Duellman, 1984)*, P. petersi* (Duellman, 1978) and *P. pustulosus* (Breder, 1946; Starret, 1960; Kenny, 1969) have been described. They differ from *P. guayaco* by lacking a discrete brown mark at tip of tail. *Physalaemus guayaco* differs further from *P. petersi* by lacking paired elliptical paravertebral glands and by having a different dorsum color pattern (Fig. 2.3).

Distribution and ecology. *Physalaemus guayaco* has been recorded from four localities in western Ecuador (Provincia del Guayas) between 32–92 m of altitude. Maximum straight-line distance between localities is 89 km (Fig. 2.6). The lowlands of western Ecuador are characterized by a low–high precipitation gradient from south to north and west to east. Precipitation is markedly seasonal south of 1° S latitude (rainy season in February–April; Lynch and Duellman, 1997). Among the known localities, annual

precipitation ranges from 420–1348 mm (15 km S Naranjal, and 11 km N Cerro Masvale, respectively) with only 27–52 mm falling during the three driest months of the year (July–September at Cerro Masvale); average annual temperature ranges between 24.6 and 25.2 C (Cerro Masvale and 15 km S Naranjal). Cerro Masvale is a private, protected reserve adjacent to a national protected area, Reserva Ecológica Manglares-Churute.

Localities are in Lowland Deciduous Costa Forest (20 km E Durán, 11 km N Cerro Masvale, 15 km S Naranjal), and Lowland Semideciduous Costa Forest (Cerro Masvale; vegetation types are as defined by Sierra et al., 1999 and Cerón et al., 1999). These vegetation types are located between 50 m and 400 m of altitude, covering 25,673 km^2 (10.3% of Ecuador's area). The forest is dryer and the terrain has lower tree densities than evergreen forests. The trees are generally below 20 m and the under-story can be dense with abundant herbaceous plants. Some tree species loss their leaves during the dry season (Cerón et al., 1999). Due to the dry conditions, amphibian diversity is low. Human impact on the natural cover has been severe in this region. More than half of the land cover is being used for agriculture and cattle rising (estimate from AEE, 2000).

All individuals were found in open areas where the original natural vegetation has been partly on completely removed by humans. At Cerro Masvale, in 20 February 2002, *P. guayaco* and *P. randi* were calling from ditches and small ponds in pastures near the buildings of Fundación Andrade field station. On 23 March 2003, only *P. randi* were calling there. However, *P. guayaco* were calling at a nearby flooded rice field (not surveyed in 2002). *Physalaemus randi* were not found at the rice field. At 11 km N Cerro Masvale, a large chorus of *P. guayaco* was calling from a flooded rice field (31 males and

1 female were collected). At the same site, a few *P. randi* were calling (two males collected). At 15 km S Naranjal, males were calling from ponds, less than 20 m away from the highway. *Scinax quinquefasciatus* was calling syntopically.

Our observations of reproductive activity took place in February 2002 and March 2003, during the rainy season. Males begin calling shortly after dusk. Males call from small ponds (down to 10 cm diameter) and ditches while floating in a few centimeters of water, usually concealed by herbaceous vegetation. Amplexus and egg deposition take place at the same sites where choruses call. *Physalaemus guayaco* constructs floating foam nests during amplexus. While the female discharges the egg masses, the male beats them with his legs to produce the foam. On 20 March 2003, a submerged pair of amplectant frogs (QCAZ 23443–44) was captured, among vegetation. Two hours after capture they were placed on a plastic container with water, they begun to build a foam nest. The male kicked the eggs with rapid movements in outbursts that lasted 2–3 seconds with intervals of approximately 10 seconds (time was measured from digital video). During the intervals, the male rested with his legs semi-extended in the foam (angle between the thigh and shank ~ 70°).

Call. Acoustic parameters of the advertisement call of five *P. guayaco* are shown in Table 2.1. The call consists of two components: (1) a single, short note consisting of 3–5 amplitude modulated pulses, followed by (2) a whine-like note (its first harmonic is a nearly pure tone with a descending frequency sweep; Fig. 2.2, Table 2.1). Although both components have harmonic structure, the harmonics are clearly defined only in the second component (up to six). One male (QCAZ 19562, call QCAZ (S) 19562; SVL =

19.15 mm) in a chorus at Cerro Masvale, was recorded at 2300 h on 19 February 2002 while calling partly submerged in 2 cm of water, 2 m from a ditch, on a grassy area (water temperature 26.6 C; air temperature 24.8 C). The average duration of the first component is 35.2% of the call (range 33.7–36.0; *n* = 10). In the first component the dominant frequency is always in the second harmonic (mean dominant frequency = 3.200 kHz; *n* = 10), whereas in the second component, the dominant frequency is always in the first harmonic (mean dominant frequency = 1.080 kHz; *n* = 10). The fundamental frequency of the first component is approximately 0.5 kHz higher than that of the second component. Measurements for calls of QCAZ 23510 (air = 28.0 C; water = 27.8 C; SVL = 16.13 mm), QCAZ 23512 (air = 27.7 C; water = 27.7 C; SVL = 16.02 mm), and QCAZ 23652 (air = 28.5 C; water = 26.6 C; SVL = 19.09 mm) are also shown in Table 2.1. As described in QCAZ 19562, in these calls the dominant frequency of the first component is always above 3 kHz (second harmonic). QCAZ 19561 (call QCAZ (S) 19561; SVL = 17.94 mm) was recorded at 2240 h on at Cerro Masvale on 19 February 2002 (air = 25.8 C; water = 25.8 C). It differs from the other calls because the dominant frequency of the first component is in the first harmonic instead of the second (Table 2.1). The average duration of the first component is 29.9% of the call (range 25.4–35.8; *n* = 18).

Antiphonal calling is frequent in male choruses. The spectrogram of such behavior is shown in Figure 2.2 (between QCAZ 23508 and a non captured individual). The frogs were recorded on 23 March 2003 at 2053 h in a flooded rice field in Cerro Masvale (air = 27.2 C; water = 27.8 C). *Physalaemus guayaco* has a call very similar to that of *P.*

montubio (Ron et al., 2004; Fig. 2.2). However, the sound frequency is slightly lower in *P. montubio* (Diagnosis).

Morphometric comparisons. Three components with eigenvalues > 1.0 were extracted from the PCA of 215 specimens belonging to four species of the *P. pustulosus* group, including *P. guayaco*. The axes accounted for 65.7% of the total variation. Along PC I, the highest loadings were for arm and tibia length. Along PC II, the highest were dorsum and head width (Table 2.4). There is a wide overlap between the morphometric space of *P. guayaco* and *P. randi* (Fig. 2.9). The overlap is less extensive between *P. guayaco* and *P. montubio*, especially along PC I (Fig. 2.9).

In the DFA classification procedure, 37 out of 55 specimens of *P. guayaco* were classified correctly. The misclassified specimens were assigned to *P. pustulatus* (nine specimens), *P. montubio* (five), and *P. randi* (four). The high rate of correct group assignment indicates some degree of morphometric distinctiveness among species, independent of overall size differences. The posterior probability for the classification of each specimen as *P. guayaco* is shown in Figure 2.10. Mean probabilities are: *P. guayaco* = 0.48 (SD = 0.20, n = 55); *P. pustulatus* = 0.22 (SD = 0.23; n = 24); *P. randi* = 0.18 (SD = 0.18; n = 35); and *P. montubio* = 0.15 (SD = 0.18; n = 101). According to the DFA of size-corrected morphology, the species most similar to *P. guayaco* is *P. pustulatus*; the least is *P. montubio*.

Phylogenetic Relationships. An expansion of the phylogenetic analysis based on morphological characters presented by Cannatella et al. (1998) shows that *P. guayaco* is part of a clade that includes the species of the *P. pustulosus* group that occur west of the

Andes in northwestern South America (Fig. 2.11); this clade is sister to a clade distributed in Central America and east of the Andes (*P. petersi* + *P. pustulosus*). The synapomorphies that support the western South American clade are (1) absence of a tarsal tubercle, and (2) narrow stalk of the alary process of the hyoid. The topology within this clade is not well supported and should be interpreted with caution because the only informative character is SVL.

The phylogenetic analysis of mtDNA (2409 bases, 348 parsimony-informative) yields a single most-parsimonious tree (Fig. 2.11). All clades have a strong support except for as a bootstrap of 67% for clade (*P. guayaco* + P. *coloradorum*). The maximum likelihood analysis (best-fit model GTR + Γ) yielded a single tree with a branching pattern identical to that from parsimony. The sister-species relationship between *P. montubio* and *P. randi* verifies the distinctiveness of *P. montubio* and *P. guayaco*, despite the similarity in external morphology and advertisement call. Low intraspecific genetic differentiation is evident in all species (uncorrected *p*-distances range 0.0004–0.0025) despite geographic distances as high as 220 km (between both populations of *P. montubio*). Genetic distances between four *P. guayaco* populations range between 0.0004 (QCAZ 23656 from 15 km S Naranjal vs. QCAZ 19561 from Cerro Masvale) and 0.0025 (QCAZ 23652 from 15 km S Naranjal vs. QCAZ 23533 from 11 km N Cerro Masvale); genetic distances between species range between 0.0972 (*P. pustulatus* QCAZ 23320 vs. *P. coloradorum* QCAZ 19418) and 0.0284 (*P. montubio* QCAZ 23190 vs. *P. randi* QCAZ 19559).

The status of the populations of *Physalaemus,* from southwestern Ecuador (Pasaje, Provincia del Oro), which have advertisement call and external morphology similar to that of *P. randi* is still unresolved. I will refer to them as *P. randi* although it is still unclear if they deserve to be considered a separate species.

2.4. DISCUSSION

Complex calls are common among species of the *P. pustulosus* group. The clade including the four smallest species (mean male SVL < 21 mm; Fig. 2.11A) is characterized by calls with shorter duration. The calls of *P. guayaco* and the recently described *P. montubio* and *P. randi* (Ron et al., 2004) are unique in having an amplitude-modulated component with several well defined pulses (first component) prior to the frequency sweep (second component) ubiquitous in members of the group (Fig. 2.2). Previous accounts of call evolution and sexual selection in the *P. pustulosus* group have referred to the first component as a "prefix" (in *P. randi* from southern Ecuador, mistakenly ascribed to *P. pustulatus*) and to the second component as a "whine" (e.g., Ryan, 1997; Ryan and Rand, 1993a; Ryan and Rand, 2001).

The spectral features of both components are different in: (1) fundamental frequency, which is 0.2–0.7 kHz higher in the first component than in the second (Table 2.1; Ron et al., 2004); and (2) sound energy distribution, which in the second component always peaks in the first harmonic (at about 0.9–1.2 kHz) but in the first component tends to be allocated equally between the first and the second harmonics (often more energy is in the second harmonic in *P. guayaco*; peak at 2.7–3.4 kHz; Table 2.1 Fig. 2.2).

The distinct spectral features of both components are likely to match the sensory sensitivity of females. Many studies have demonstrated a correlation between auditory tuning and the frequency of the bands with high-energy allocation in conspecific advertisement calls (reviewed by Gerhardt and Schwartz, 2001). The auditory tuning of *Physalaemus* shows two spectral ranges of enhanced sensitivity, one from 0.1 to 1.100 kHz, which presumably represents amphibian papilla tuning, and another from 2.100 to 2.550 kHz (presumably basilar papilla tuning; Wilczynski et al., 2001, based on measurements on *P. petersi, P. pustulosus, P. coloradorum,* and *P. randi*). Studies of sexual selection in *P. pustulosus* suggest that matching between sound frequency of the advertisement call and female auditory tuning may influence female mate choice (Ryan and Rand, 1999). *Physalaemus pustulosus* has a complex advertisement call with two main components: (1) a whine-like frequency sweep (likely homologous to the second component of *P. guayaco, P. montubio and P. randi*) that stimulates primarily the amphibian papilla; and (2) a subsequent and facultative higher frequency "chuck" (peak energy at 2.55 kHz) that stimulates the basilar papilla (Ryan et al., 1990). The whine is necessary and sufficient to attract females. However, females prefer calls that have a whine and one (or more) chucks to whine-only calls (Rand and Ryan, 1981). It has been suggested that females prefer the whine-chuck calls because they are a better match for the spectral sensitivity of the amphibian and basilar papillae (Wilczynski et al., 2001).

We propose that the first component of the calls of *P. guayaco, P. montubio,* and *P. randi* have spectral features that stimulate the basilar papilla in a similar manner to the chuck of *P. pustulosus. I* base this prediction on the following evidence: (1) in several

species of anurans (including *P. pustulosus*) there is a match between auditory tuning and the frequencies emphasized in the conspecific advertisement call (Gerhardt and Schwartz, 2001); (2) the frequency of the second harmonic of the first component is close to the enhanced sensitivity range of the basilar papilla (2.549 kHz in *P. randi* from Pasaje, Ecuador; Wilczynski et al., 2001 [referred as "*P. pustulatus*" in that publication]); (3) the peak frequency of the second harmonic of the first component is similar to that of the "chuck"; and (4) females of *P. pustulosus* prefer their conspecific whines with an artificially added first component (from calls of *P. randi* from Pasaje) over whine-only calls, suggesting that the first component may play a functional role similar to that of the "chuck" (Ryan and Rand, 1993a). In other words, the first component of the calls of *P. guayaco*, *P. montubio*, and *P. randi* may be functionally similar to the "chuck" of the complex calls of *P. pustulosus* because it has a significant amount of energy that should match the spectral sensitivity of the basilar papilla. As in *P. pustulosus*, the second component has most of its energy matching the spectral sensitivity of the amphibian papilla. If in fact females prefer calls that match the frequency sensitivity of both papillae, I might expect that female *P. guayaco*, *P. montubio* and *P. randi* will show a preference for complex calls (chuck + second component or first component + second component) over simple calls (second component only).

Physalaemus coloradorum also belongs to the clade of small species. The following discussion is based on averages from advertisement calls of five male *P. coloradorum* from 5 km NW La Florida, Provincia del Pichincha (QCAZ 19412, 19416–8, and 19441). The call of *P. coloradorum* has two components that resemble those of the

calls of *P. guayaco, P. montubio,* and *P. randi* (Fig. 2.2). The first component in *P. coloradorum* contains about 65% of the energy of the call and also is amplitude-modulated. In contrast with *P. guayaco, P. montubio* and *P. randi,* the pulses of the first component are weakly defined in *P. coloradorum* (Fig. 2.2). As in all other members of the species group, the second component is a downward frequency sweep. The dominant frequency of the first component is approximately 0.149 kHz higher than that of the second. Although the dominant frequency of the first component is in the first harmonic, at 1.104 kHz, 45% of its sound energy is allocated above 1.2 kHz and therefore should stimulate the basilar papilla. In fact, the frequency with the most energy in the second harmonic of the first component (2.227 kHz) matches the best excitatory frequency of the basilar papilla (2.228 kHz; Wilczynski et al. 2001). It is unknown whether this level of stimulation of the basilar papilla can increase the attractiveness of the calls to females although this scenario is plausible considering that the energy > 1.2 kHz in the first component is 26% of the total energy of the call of *P. coloradorum* compared to 7% for the chuck of *P. pustulosus* (Ryan and Rand, 1990). Female *P. coloradorum* prefer conspecific calls to which chucks of *P. pustulosus* have been artificially appended versus normal conspecific calls (Ryan and Rand, 1993b).

It has been assumed that the advertisement calls of the South American *Physalaemus* distributed west of the Andes are characterized by being simple, with a dominant frequency < 1.0 kHz, and very little energy in the range of sensitivity of the basilar papilla (e.g., Ryan, 1990; Ryan and Rand, 1993a; Wilczynski et al. 2001). However, the calls of the recently described species (herein and in Ron et al. 2004) and

even that of *P. coloradorum* have a complex structure consisting of two components with different spectral features and a significant amount of energy in the range of frequency sensitivity of both the amphibian and basilar papillae. These new findings make necessary a reevaluation of previous species-level analyses of call evolution and female choice that have assumed that calls with high frequency components are restricted to the clade (*P. pustulosus* + *P. petersi*). This reevaluation is persued in chapters 4 and 5 of this dissertation.

Table 2.1. Call parameters of *Physalaemus guayaco* (ranges in parentheses). The call of *P. guayaco* consists of one note with amplitude-modulated pulses (first component) followed by a "whine" like note with a frequency sweep (second component). Specimen catalog numbers at the Museo de Zoología de la Pontificia Universidad Católica del Ecuador (QCAZ) are shown. Sample sizes are number of calls. See text for details.

Specimen	Mean call duration (ms)	Call repetition rate (calls/min)	Mean interval between calls (ms)	Rise time of the first component (ms)	Mean dominant frequency of the first component (kHz)	Mean fundamental frequency of the first component (kHz)	Mean fundamental frequency of the second component (kHz)
QCAZ 19561	68.2	221.2	203	15.1	1.372	1.372	1.151
n = 18	(56.7–76.0)		(161.9–405.1)	(9.1–20.8)	(1.281–1.491)	(1.281–1.491)	(1.125–1.179)
QCAZ 19562	70.6	220.9	201	17.7	3.200	1.577	1.079
n = 10	(67.5–72.8)		(188–224)	(16.5–18.8)	(3.149–3.273)	(1.394–1.679)	(1.060–1.098)
QCAZ 23510	52.8	259.6	178.3	14.3	3.237	1.789	1.066
n = 10	(49.1–54.9)		(154.1–246.6)	(8.5–15.6)	(3.168–3.341)	(1.744–1.880)	(1.064–1.097)
QCAZ 23512	65.3	259.1	166.3	19.0	3.359	1.511	1.091
n = 10	(61.9–68.8)		(153.7–188.1)	(10.4–23.5)	(3.274–3.442)	(1.472–1.703)	(1.070–1.120)
QCAZ 23652	69.4	259.9	190.5	17.9	3.237	1.631	1.048
n = 15	(64.6–74.9)		(150.6–270.5)	(10.5–24.4)	(3.004–3.325)	(1.634–1.907)	(1.007–1.084)

Table 2.2. Descriptive statistics for morphometric measurements of male *Physalaemus guayaco* from three localities in Provincia del Guayas, Ecuador. Mean ± SD is given with range below. Bold figures are combined for males of all populations. Abbreviations are: SVL = snout-vent length; DW = dorsum width; TL = tibia length; FL = femur length; AL = arm length; HL = head length; HW = head width; EN = eye-nostril distance. All measurements are in mm.

	SVL	DW	TL	FL	AL	HL	HW	EN
1.1. *P. guayaco* (*n* = 55)	**16.85 ± 0.99**	**6.20 ± 0.39**	**7.86 ± 0.42**	**7.56 ± 0.51**	**3.9 ± 0.22**	**5.79 ± 0.28**	**5.51 ± 0.32**	**1.83 ± 0.20**
Cerro Masvale	17.02 ± 1.01	6.37 ± 0.23	7.95 ± 0.40	7.72 ± 0.50	3.85 ± 0.24	5.76 ± 0.32	5.57 ± 0.34	1.84 ± 0.19
(*n* = 20)	15.31–19.15	5.91–6.83	7.46–8.91	6.96–8.92	3.41–4.36	5.32–6.41	5.08–6.41	1.54–2.18
11 km N Cerro Masvale	16.57 ± 0.75	6.04 ± 0.36	7.74 ± 0.36	7.37 ± 0.46	3.90 ± 0.17	5.76 ± 0.22	5.46 ± 0.29	1.83 ± 0.22
(*n* = 30)	15.51–19.10	5.57–7.35	7.13–8.81	6.32–8.52	3.50–4.16	5.30–6.27	5.07–6.45	1.44–2.34
15 km S Naranjal	17.84 ± 1.46	6.68 ± 0.48	8.24 ± 0.58	8.06 ± 0.38	4.14 ± 0.25	6.07 ± 0.33	5.54 ± 0.44	1.83 ± 0.12
(*n* = 5)	16.04–19.38	6.25–7.37	7.51–9.10	7.58–8.45	3.75–4.36	5.57–6.39	4.92–6.06	1.64–1.94

Table 2.3. Measurements (in mm) of developmental stages (*sensu* Gosner, 1960) of four tadpoles of *Physalaemus guayaco* sp. nov. Abbreviations are: TL = total length; BL = body length; BW = body width; BH = body height; TAL = tail length; ED = eye diameter; ODW = oral disc width; IOD = interorbital distance (measured between centers of pupils); IND = internarial distance (measured between centers of narial apertures); MTH = maximum tail height; TMH = tail muscle height; TMW = tail muscle width.

Variable	Stage			
	A (28)	B (29)	C (29)	D (31)
TL	13.3	16.2	18.0	18.9
BL	4.9	6.0	7.0	7.1
BW	3.7	4.8	4.9	5.0
BH	2.8	3.4	3.7	4.0
TAL	8.3	10.2	11.1	11.9
ED	0.7	0.8	0.9	1.0
ODW	1.4	1.6	1.6	1.8
IOD	1.7	2.1	2.2	2.4
IND	0.9	1.1	1.2	1.1
MTH	2.6	3.2	3.7	3.3
TMH	1.3	1.4	1.7	2.1
TMW	0.9	1.3	1.4	1.4

Table 2.4. Character loading and percentage of explained variance for Principal Components (PC) I–III for seven morphometric variables. To remove the effect of "size", linear regressions were performed between all variables and snout-vent length (SVL). The PC analysis was applied to the residuals from the regressions. Bold figures indicate highest loadings.

Variable	Size-free morphology		
	PC I	PC II	PC III
Residual dorsum width	0.347	**0.737**	–0.329
Residual tibia length	**0.685**	–0.522	0.014
Residual femur length	0.622	–0.115	–0.069
Residual arm length	**0.685**	–0.349	–0.183
Residual head length	0.540	0.046	0.449
Residual head width	0.473	0.701	–0.196
Residual eye-nostril distance	0.153	0.364	**0.810**
Eigenvalue	1.984	1.576	1.043
%	28.3	22.5	14.9

Figure 2.1. Dorsolateral and ventral views of *Physalaemus guayaco* from 15 km S Naranjal, Provincia del Guayas, Ecuador: (A) QCAZ 23531, adult female, SVL = 18.26; (B) 23510, adult male, SVL = 16.13.

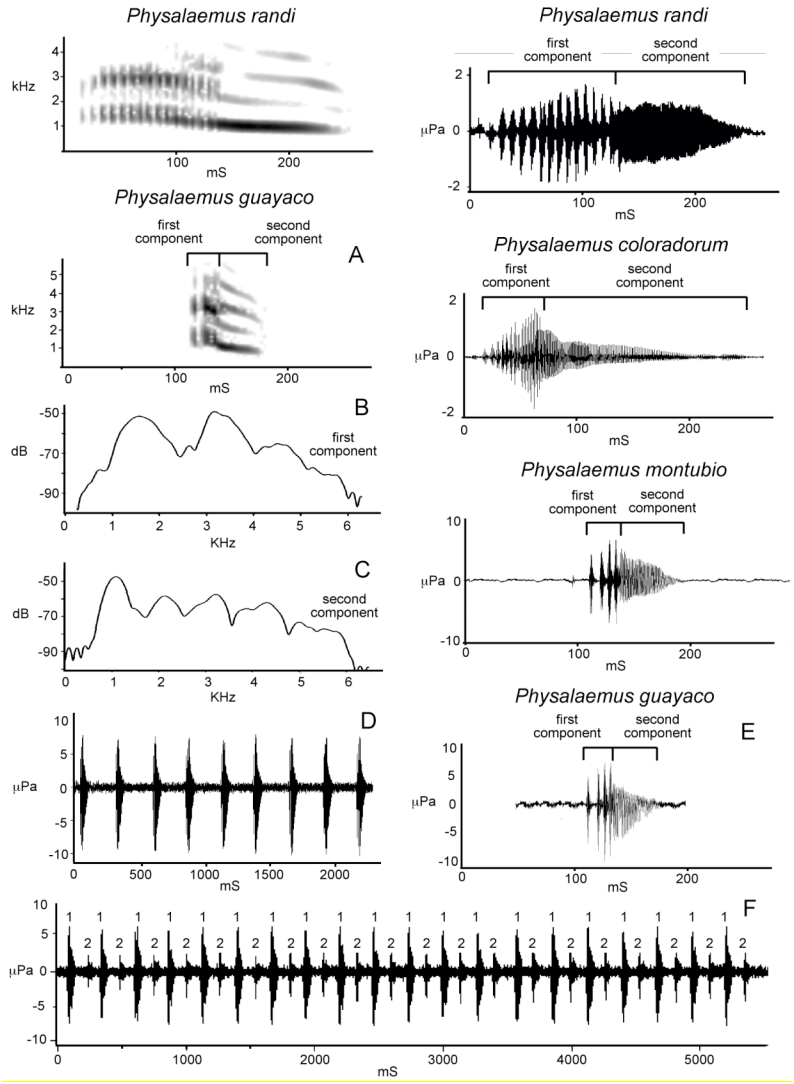

Figure 2.2. Calls of *Physalaemus* from western Ecuador. Letters are for *P. guayaco* (A–E from QCAZ 19562; F from QCAZ 23508 and uncollected male) recorded at Cerro Masvale, Provincia del Guayas, Ecuador. (A) Sonogram of single call, (B) power spectrum of the first call component, (C) power spectrum of the second call component, (D) oscillogram of series of calls, (E) oscillogram of single call, (F) oscillogram of the antiphonal calls of two males. Calls from QCAZ 23508 are marked "1"; calls from a non-collected male are marked "2". The first component is a series of amplitude-modulated pulses; the second component is a whine-like note, its first harmonic is a nearly pure tone. There is a switch in the dominant frequency between the first and the second component. The first and second components also are evident in the oscillograms of the advertisement calls of *P. randi* (QCAZ 19752, Cerro Masvale), *P. coloradorum* (QCAZ 19412, 5 km NW La Florida, Provincia del Pichincha), *P. montubio* (QCAZ 23214, San Vicente, Provincia de Manabí) shown in the right column.

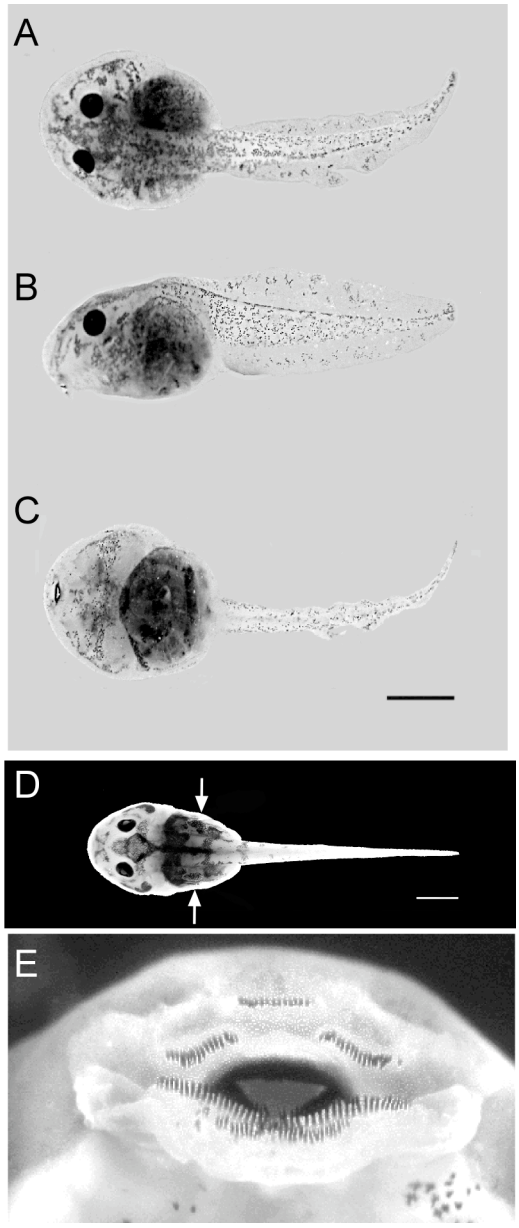

Figure 2.3. Tadpole of *Physalaemus guayaco*, QCAZ 24006A: (A) dorsal, (B) lateral, and (C) ventral views. (D) Tadpole of *Physalaemus petersi* (Stage 35, QCAZ 18282); arrows point to paravertebral glands. (E) Mouth parts of the tadpole of *P. guayaco*, QCAZ 24006B.

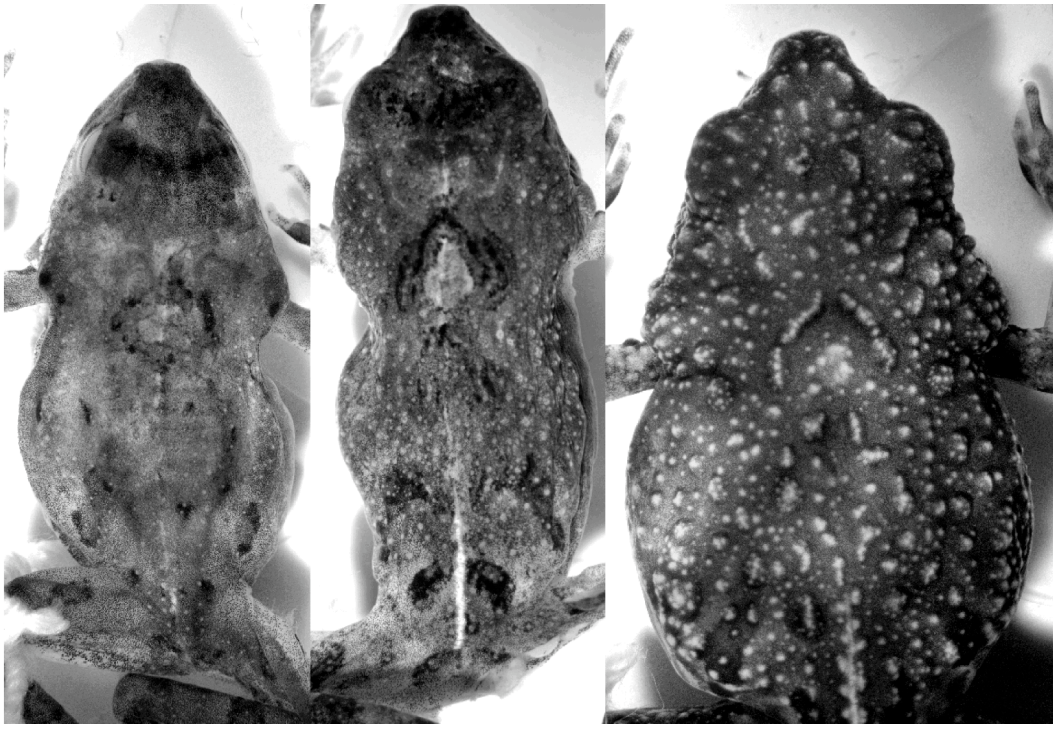

Figure 2.4. Dorsal photographs of adult *Physalaemus guayaco* (A; QCAZ 23510), *P. randi* (B; QCAZ 23579), and *P. coloradorum* (C; QCAZ 2975) showing differences in skin texture. Note that *P. guayaco* has fewer and smaller dorsal tubercles.

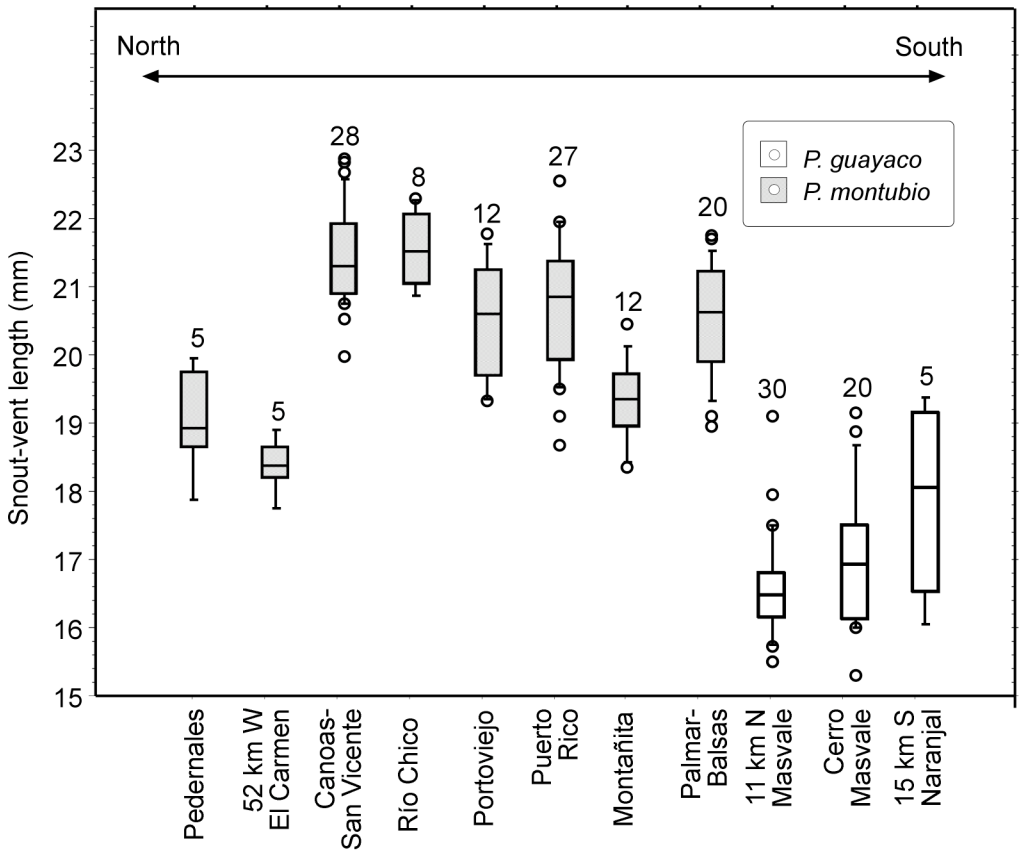

Figure 2.5. Box plot of the snout-vent length of males eight populations of *P. montubio* (117 specimens) and three populations of *P. guayaco* (55 specimens) from western Ecuador. (See Figure 2.8 for locality map.) Sample sizes for each locality are shown on top of the box. Sequence of localities from left to right matches their relative geographic position from north to south.

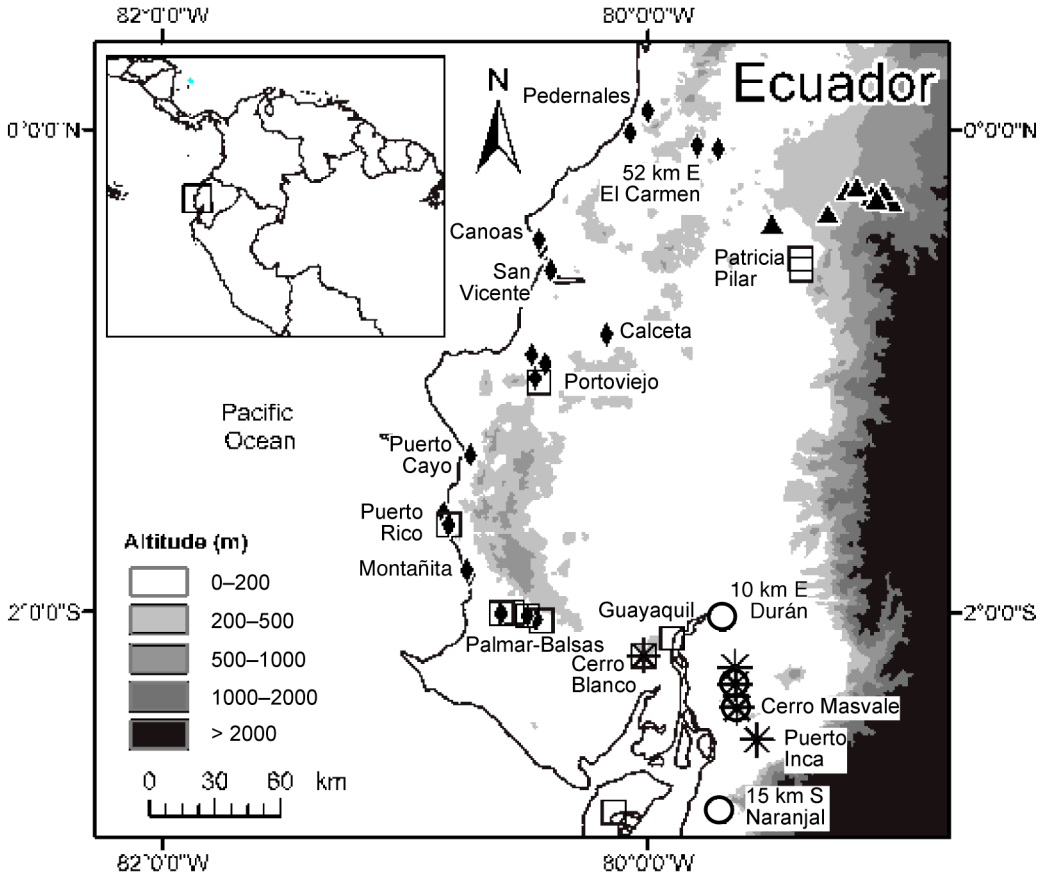

Figure 2.6. Known records of *Physalaemus coloradorum* (triangles), *P. guayaco* (circles), *P. montubio* (diamond), *P. pustulatus* (squares), and *P. randi* (asterisks). Locality data are based on specimens deposited in American Museum of Natural History, California Academy of Sciences, Museo de Zoología de la Pontificia Universidad Católica del Ecuador, Museum of Comparative Zoology Harvard University, National Museum of Natural History, and Natural History Museum University of Kansas (Supplemental Data 2.1).

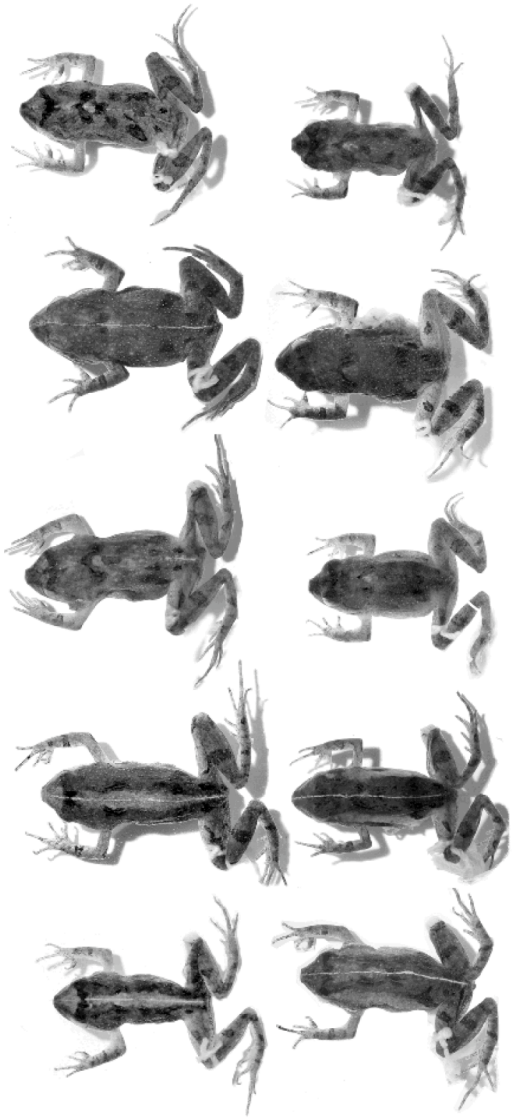

Figure 2.7. Dorsal views of adult *Physalaemus guayaco* showing variation in dorsal patterns. Left to right, upper row: QCAZ 23656 (male), QCAZ 23654 (female), QCAZ 23574 (male), QCAZ 23445 (male), QCAZ 23576 (male); lower row: QCAZ 23652 (male), QCAZ 23531 (female), QCAZ 23561 (male), QCAZ 23557 (male), QCAZ 23534 (male). All from Provincia del Guayas, Ecuador. (See Supplemental Data 2.1 for locality data.)

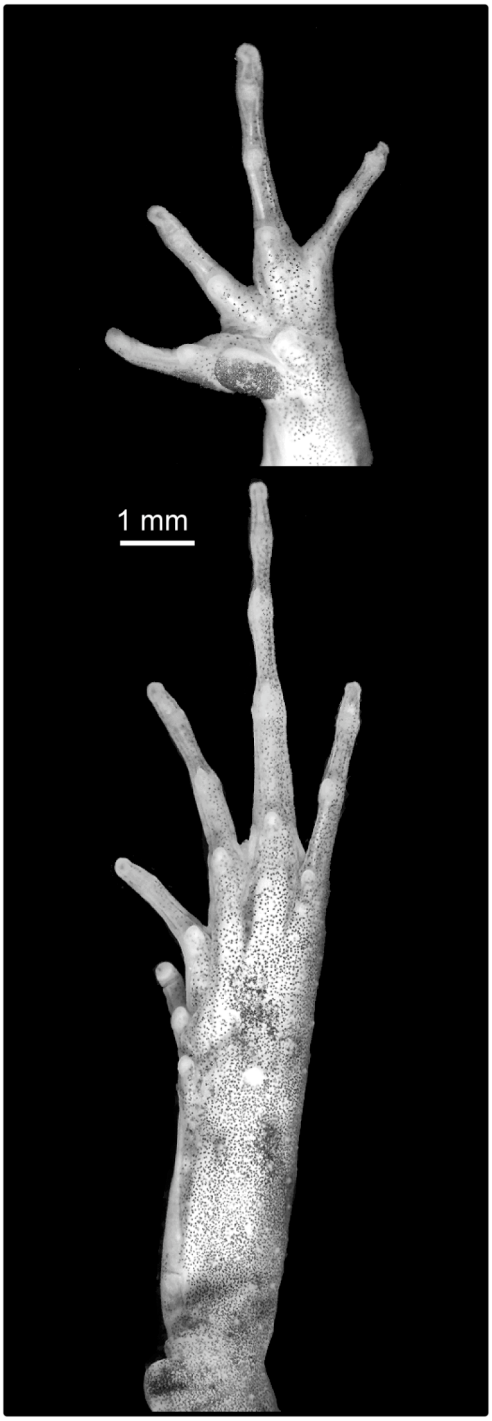

Figure 2.8. Ventral views of the left hand and foot of the paratopotype of *Physalaemus guayaco*, QCAZ 23515 (adult male from Cerro Masvale, Ecuador; SVL = 17.45).

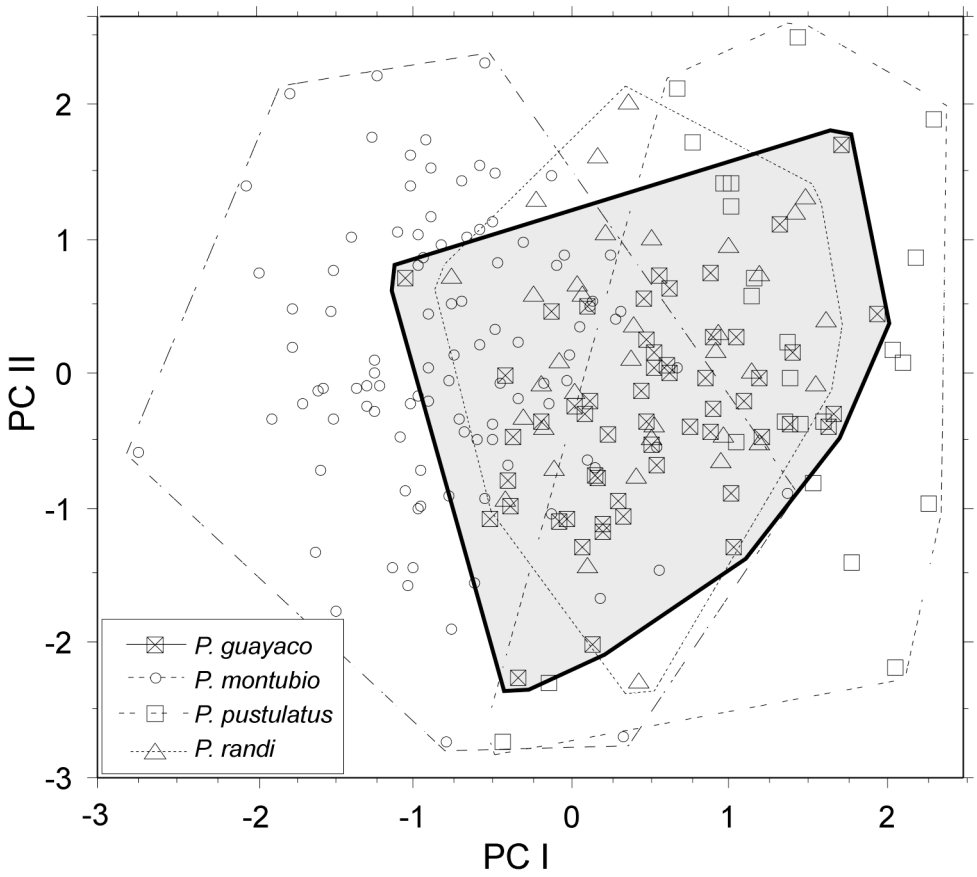

Figure 2.9. Axes I and II from Principal Components Analysis based seven size-corrected morphological variables for *Physalaemus guayaco* (55 specimens), *P. montubio* (101), *P. pustulatus* (24), and *P. randi* (35).

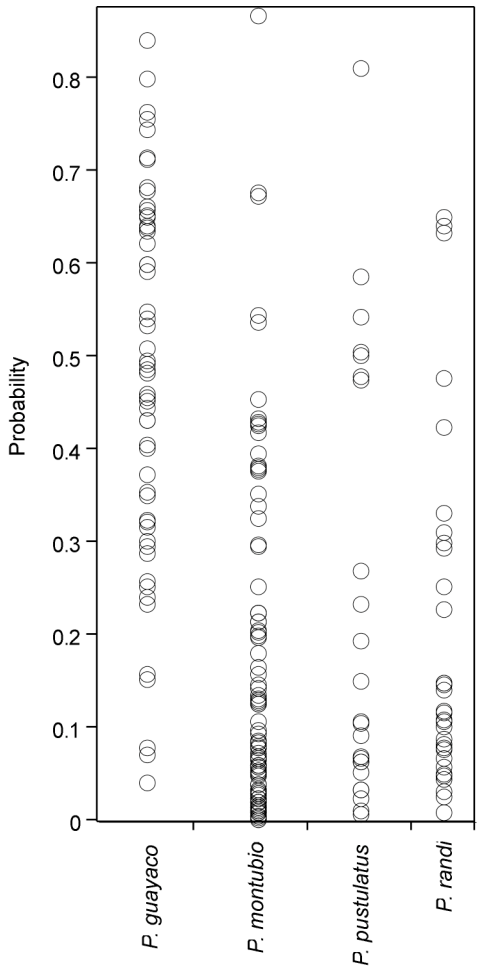

Figure 2.10. Posterior probabilities of the assignment to *Physalaemus guayaco* of each of 101 specimens of *P. montubio*, 24 of *P. pustulatus*, 35 of *P. randi*, and 55 of *P. guayaco* derived from Discriminant Function Analysis of seven size-corrected morphometric variables. *Physalaemus montubio* specimens have the lowest average probabilities of being misclassified as *P. guayaco* (in 70.3% $P < 0.20$).

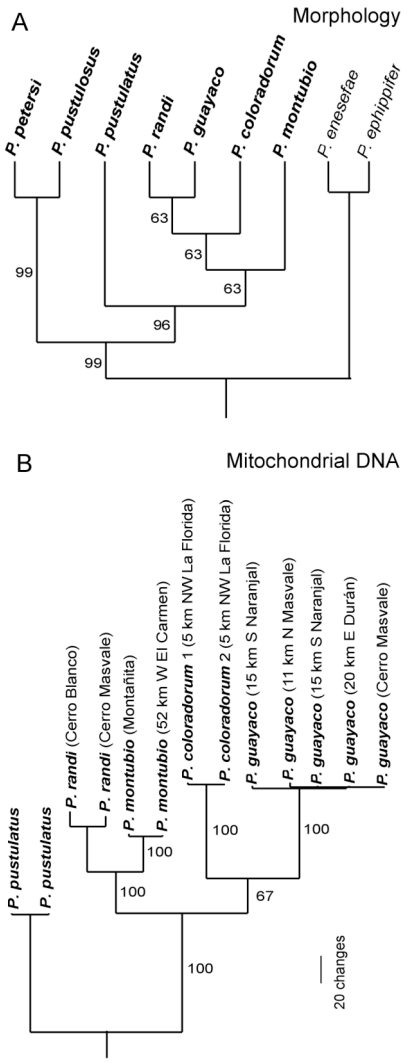

Figure. 2.11. (A) Single, most parsimonious tree of the phylogenetic relationships of the *Physalaemus pustulosus* species group (boldface taxa) based on 13 morphological characters (tree length = 13.31 [raw TL = 13299], CI = 0.976, consistency index [excluding uninformative characters] = 0.975, RI = 0.543). Raw tree length is divided by 999 because of the use of a step-matrix to code one morphometric character (scaled to 999; Supplemental Data 2.2 for details). (B) Single, most parsimonious rooted phylogram (length of branches proportional to the amount of estimated change for that lineage) based on 2409 bases of mtDNA genes12S rRNA, valine-tRNA gene, and 16S rRNA (TL = 455, consistency index = 0.888, CI [excluding uninformative characters] = 0.884, RI = 0.935). In (A) and (B), numbers next to internal branches are nonparametric bootstrap support values (from 1000 iterations). Maximum likelihood analysis (best-fit model GTR + Γ) yielded a single tree (not shown) with identical topology to that from parsimony.

Supplemental Data 2.1. Examined Specimens.

Cleared-and-stained specimens are designated with C&S.

Physalaemus coloradorum: ECUADOR: PROVINCIA DEL PICHINCHA: Tinalandia (AMNH 111556); 1–5 km NW from La Florida, 1003 m (QCAZ 2975, 19373–74, 19417–18, 19439–41); Near Alluriquín (QCAZ 19336); on road between Alluriquín and Santo Domingo de los Colorados (QCAZ 19294); Santo Domingo de los Colorados (KU 187271); 6 km E Santo Domingo de los Colorados (USNM 212256); Río Baba, 5–10 km SSW Santo Domingo de los Colorados (KU 146194); Río Cupa (USNM 196869).

Physalaemus montubio: ECUADOR: PROVINCIA DE MANABÍ: Pedernales, 85 m (QCAZ 23197–3201); Estero Ancho, 329 m (QCAZ 23188–192); Río Chico, 24 m (QCAZ 23252–59); road between Canoas and San Vicente, 10–20 m (QCAZ 23204–05, 23207–09, 23231–36); San Vicente, 10 m (QCAZ 23210, 23212, 2321418, 23220–24, 23226–30); Portoviejo, 56 m (QCAZ 23237–247, 23249); Puerto Rico, 30 m (QCAZ 19366, 19375, 19377–380, 19511, 19515–17, 19519–522, 19524 [holotype], 19526–527, 19530–33, 19549–550, 19552, 1955557). PROVINCIA DEL GUAYAS: Montañita, 20 m (QCAZ 23271–282); on road between El Palmar and Balsas, 5–110 m (QCAZ 23323, 23325, 23330–31, 23373–77, 23379–81, 23386–392, 23397).

Physalaemus petersi: BOLIVIA: COCHABAMBA: 6.5 km N Chipiriri, 260 m (KU 135513–16). ECUADOR: PROVINCIA DE ORELLANA: Estación Científica de la Universidad Católica del Ecuador, Parque Nacional Yasuní, 240 m (QCAZ 14733–38, 18282 [tadpole]).

Physalaemus pustulatus: ECUADOR: PROVINCIA DE MANABÍ: Puerto Rico, 10 m (QCAZ 19355, 19513–14, 19518, 19523, 19537 [C & S]), 19541–42, 19545–48, 19551, 19553–54. PROVINCIA DE LOS RÍOS: Patricia Pilar, 200 m (QCAZ 19538–40, 19605–14, 19745–47, 19748 [C & S]). PROVINCIA DEL GUAYAS: Guayaquil (MCZ 7666 [holotype]); Isla Puná (CAS 5408); Cerro Blanco (QCAZ 23427).

Physalaemus randi: ECUADOR: PROVINCIA DEL GUAYAS: 11 km N Cerro Masvale, 40 m (QCAZ 23461, 23523); Cerro Masvale, 92 m (19558–560, 19563 [holotype], 19564–66, 19569, 19571, 19573–75, 19576 [C & S], 19577–78, 19579–582 [all C & S], 19585–88, 19590–91, 19597, 19752–55); Puerto Inca, 23489–493.

Supplemental Data 2.2. Data Matrix for Morphology-Based Phylogenetic Analysis.

	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*
P. petersi	1	0	1	1	1	1	1	1	1	0	0	1	1
P. pustulatus	0	1	1	1	1	0	0	1	0	1	1	0	2
P. pustulosus	1	0	1	1	1	1	0	1	1	0	0	1	3
P. randi	0	1	1	1	1	0	0	1	0	1	1	0	4
P. montubio	0	1	1	1	1	0	0	1	0	1	?	0	5
P. guayaco	0	1	1	1	1	0	0	1	0	1	?	0	6
P. coloradorum	0	1	1	1	1	0	0	1	0	1	1	0	7
P. enesefae	0	0	0	0	0	0	0	0	0	0	0	0	8
P. ephippifer	0	0	0	0	0	0	0	0	0	0	0	0	9

Character 13 (stepmatrix)

	0	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
0	--	998	740	784	19	272	0	258	606	918
1	998	--	258	214	979	726	998	740	392	80
2	740	258	--	44	721	468	740	482	134	178
3	784	214	44	--	765	512	784	526	178	134
4	19	979	721	765	--	254	19	239	588	899
5	272	726	468	512	254	--	272	15	334	646
6	0	998	740	784	19	272	--	258	606	918
7	258	740	482	526	239	15	258	--	349	660
8	606	392	134	178	588	334	606	349	--	312
9	918	80	178	134	899	646	918	660	312	--

Supplemental Data 2.3. Locality data and GenBank accession numbers for *Physalaemus* included in the phylogeny based on DNA mitochondrial sequences. All specimens were collected in Ecuador and are deposited at the collection of the Museo de Zoología, Pontificia Universidad Católica del Ecuador (QCAZ).

Species	QCAZ Museum No.	Locality	GenBank Accession No.
P. coloradorum (1)	19417	AY834181	Pichincha: 5 km NW La Florida
P. coloradorum (2)	19418	AY834182	Pichincha: 5 km NW La Florida
P. guayaco	19561	AY834172	Guayas: Cerro Masvale
P. guayaco	23445	AY834173	Guayas: 11 km N Cerro Masvale
P. guayaco	23533	AY834175	Guayas: 11 km N Cerro Masvale
P. guayaco	23652	AY834176	Guayas: 15 km S Naranjal
P. guayaco	23656	AY834174	Guayas: 15 km S Naranjal
P. montubio	23190	AY834177	Manabí: Estero Ancho, 52 km W El Carmen
P. montubio	23271	AY834178	Guayas: Montañita
P. pustulatus	19551	AY834183	Manabí: Puerto Rico
P. pustulatus	23320	AY834184	Guayas: near Balsas
P. randi	19559	AY834179	Guayas: Cerro Masvale

P. randi	23425	AY834180	Guayas: Cerro Blanco

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Chapter 3

Phylogeny of the túngara frog genus *Engystomops* (= *Physalaemus pustulosus* species group; Anura: Leptodactylidae)*

Abstract. I present a phylogeny of the Neotropical genus *Engystomops* (= *Physalaemus pustulosus* species group) based on sequences of ~2.4 kb of mtDNA, (12S rRNA, valine-tRNA, and 16S rRNA) and propose a phylogenetic nomenclature. The phylogeny includes all described taxa and two unnamed species. All analyses indicate that *Engystomops* is monophyletic and contains two basal allopatric clades. Clade I (Edentulus) includes *E. pustulosus* and the Amazonian *E. petersi* + *E.* cf. *freibergi*. Clade II (Duovox) includes all species distributed in W Ecuador and NW Peru. Brevivox, a clade of small-sized species is strongly supported within Duovox. Populations of *Engystomops pustulosus* fall into two well-supported clades, each of which occupies two disjunct portions of the species range. Overall, my phylogeny is congruent with most previous hypotheses. This study is among the few published species-level phylogenies of Neotropical amphibians derived from molecular datasets. A review of the proportion of new species detected by similar studies suggests that the increasing use of molecular techniques will lead to the discovery of a vast number of species of Neotropical amphibians.

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3.1 INTRODUCTION

Physalaemus and *Engystomops* are closely related genera of frogs of the subfamily Leptodactylinae; until recently these were allocated into a single genus (*Physalaemus*) with 49 species (updated from Frost, 2004) and four species groups (Lynch, 1970; Cannatella and Duellman, 1984): *P. biligonigerus*, *P. cuvieri*, *P. pustulosus*, and *P. signifer* group. In a taxonomic review, Nascimento et al. (2005) resurrected the genus *Engystomops* for the species of the *P. pustulosus* group and defined seven species groups within *Physalaemus*. *Engystomops* is distributed from central Veracruz (Mexico) to the Amazon Basin and the lowlands of western Ecuador and NW Peru.

Engystomops has been a model system in studies of sexual selection and animal communication since the 1980's (e.g., Ryan, 1983; Ryan and Drewes, 1990; Ryan and Rand, 1995; Cannatella et al., 1998; Bosch et al., 2000; Wilczynski et al., 2001; Tarano and Ryan, 2002). The systematics of *Engystomops* was reviewed by Cannatella and Duellman (1984), who recognized four species and provided morphological evidence for the group's monophyly. Sister species status was established for (*E. petersi* + *E. pustulosus*) and (*E. coloradorum* + *E. pustulatus*). Ryan and Rand (1993) presented a phylogeny based on unpublished morphological characters, allozyme variation and 12S mtDNA sequences (Fig. 3.1B). Their phylogeny differed from that implied by Cannatella and Duellman (1984) in placing *E. pustulosus* as sister taxon to the remaining species instead of to *E. petersi*. Cannatella et al. (1998) included two additional species and analyzed morphology, behavior, allozyme variation and 12S rRNA and COI mtDNA sequences. The combined analysis of all characters placed *E. pustulosus* as sister taxon to

the clade (*E. petersi* + *E.* cf. *freibergi*). However, their COI mtDNA data partition supported *E. pustulosus* as sister taxon to all species of the group (Fig. 3.1C-D). A recent phylogeny based on COI mtDNA shows the same basal position for *E. pustulosus* (Weigt et al., 2005). To demonstrate the taxonomic status of the cryptic *E. guayaco*, Ron et al. (2005) included a brief phylogeny based on a subset of the mtDNA data presented here (five species). Because that analysis is congruent with my results, I will not discuss it further.

Taxon sampling influences tree topology (Zwickl and Hillis, 2002) and the interpretation of character evolution (Ackerly, 2000). Comprehensive taxon sampling for phylogenetic inference is particularly important in model systems, like *Engystomops*, where large datasets need to be analyzed in an evolutionary framework. The earliest studies on communication and sexual selection in *Engystomops* had the virtue of being among the first comparative analyses of behavioral characters that used explicit phylogenetic methods (e.g., Ryan and Rand, 1995). Unfortunately, the phylogenies used in those studies have incomplete taxon sampling and/or conflicting topologies (Fig. 3.1). For example, the influential concept of the Sensory Exploitation Hypothesis, which posits that a male secondary sexual trait can evolve to take advantage of pre-existing female sensory biases (Ryan, 1990) was based on character reconstructions on a phylogeny that only included the four species of *Engystomops* known at the time (Fig. 3.1A). Since then, the number of species of *Engystomops* has more than doubled. The addition of these new taxa could plausibly compromise support for the Sensory Exploitation Hypothesis,

depending on the resulting new topology and character state distributions of the male secondary sexual trait and female mate choice in the added species.

The existence of undescribed species of *Engystomops* has been previously reported (e.g., Cannatella et al., 1998; Ryan and Rand, 2001) and recent fieldwork has confirmed and expanded the list of new species (Ron et al., 2004, 2005). The present study is an effort to provide a complete phylogeny for all extant species of *Engystomops* (described as well as new, but as yet undescribed, species; 10 or 11 in total). The phylogeny is based on analyses of ~2.4 kb from three mitochondrial genes (ribosomal RNA genes, and the valine tRNA gene). In combination with the wealth of available data on call evolution and female mate preference, this new phylogeny presents new opportunities to expand, complement, and reevaluate previous analyses of sexual selection and the evolution of communication in this model clade.

As exemplified by the recent discovery of morphologically cryptic species in *Engystomops* (Ron et al., 2004, 2005), the use of genetic markers in systematics has an enormous potential to facilitate the global inventory of biodiversity. The revolution that systematics is experiencing will be crucial for management and conservation of biotic resources considering that probably < 10% of species on the planet have been discovered and as few as < 1% are known beyond a succinct anatomical description (Wilson, 2005). Although the taxonomic deficit seems to be less severe among terrestrial vertebrates, sampling of amphibians and reptiles inhabiting highly diverse regions in the Neotropics is far from complete (Duellman, 1999; Rodrigues, 2005). Although it is clear that the inventory of species of tropical amphibians and reptiles is still inadequate, the extent of

this inadequacy is unknown. In this paper, I also combine my results with those from other species-level phylogenies of Neotropical amphibians to estimate the potential impact of molecular systematics on the discovery of new species in the tropics.

3.2 MATERIALS AND METHODS

Taxa sampled. I sampled 36 populations of *Engystomops* from throughout the Neotropical Region belonging to 8 described and at least 2 unnamed species (Table 3.1; Fig. 3.2). Tissue samples (liver and muscle) were stored in 95% ethanol, tissue buffer, or DMSO buffer. Sample sources and sequence accession numbers are listed in Table 3.1. All available information suggests that *Engystomops* is monophyletic and sister to *Physalaemus* (Cannatella and Duellman, 1984; Tarano and Ryan, 2002; Nascimento et al. 2005; Ron et al.,2005). The monophyly of *Engystomops* has been corroborated by phylogenetic analyses of ~2400 bp of mtDNA that included 25 species of *Physalaemus* (DCC, unpublished data). For the outgroup, I used six species representing all three remaining species groups recognized in *Physalaemus* (groups as defined by Lynch, 1970): (1) *P. albonotatus*, *P. barrioi*, and *P. enesefae* (= *P. fischeri*) from the *P. cuvieri* group; (2) *P. biligonigerus* and *P. nattereri* from the *P. biligonigerus* group; and (3) *P. signifer* from the *P. signifer* group. For ease of comparison, my nominal species *E.* sp. B is the same as "*P.* sp. B" in Cannatella et al. (1998). In Cannatella et al. (1998) two species were misnamed: "*Physalaemus pustulatus*" is in fact *E. randi* whereas "*P.* sp. C" is *E. pustulatus.*

DNA extraction, amplification, purification, and sequencing. Total DNA was extracted from muscle and liver tissue preserved in ethanol and tissue storage buffer using Dneasy (Qiagen Corp.) or Viogene DNA extraction kits. Polymerase chain reaction (PCR) was used to amplify a 2.4-kb region that included 12S rRNA, valine-tRNA, and 16S rRNA genes. I amplified the segment using 4 to 6 overlapping DNA fragments using primers listed in Darst and Cannatella (2004). I use the following combination of eight primers to amplify four overlapping PCR products of ≈600 bp (notation and number follows Goebel *et al*., 1999 and publications above): MVZ59 (#29)-tRNAval (#73); L1091 (#46)-16SH; 12SM-16Sar (#88); and 16SC-16Sbr (#96). All PCR products were amplified under standard conditions and with the following PCR profile: (1) initial heating for 2 min at 94°C; (2) 37 cycles of: 94°C for 30 s, 45–48°C for 30 s and 72°C for 60 s; and (3) final extension for 8 min at 72°C. PCR products were visualized on an agarose/TBE gel and single fragments were excised and purified using QIAquick (Qiagen Corp.) and Gel-M (Viogene Corp.) gel extraction kits following the manufacturer's specifications. Purified PCR products were sequenced in both directions using ABI Prism BigDye Terminator chemistry (versions 2.0 and 3.0; Applied Biosystems, Inc.). Sequenced products were cleaned using CentriSep columns (Princeton Separations, Corp.) with Sephadex G-50 (Sigma-Aldrich, Corp.) and then run on a capillary automated sequencer (ABI 3100; Applied Biosystems, Inc.). Sequences were edited for ambiguities and sequence errors using Sequencher (versions 4.1 and 4.2; Gene Codes Corp.). A continuous sequence was generated from the 8–12 overlapping fragments obtained.

Sequence alignment and analyses. *I* analyzed 2422 bp of the mitochondrial genes12S rRNA, valine-tRNA, and 16S rRNA. Preliminary alignment was done with CLUSTAL X 1.8 (Thompson et al., 1997). The sequence matrix was imported to MacClade (version 4.06; Maddison and Maddison, 2000) and the ambiguously aligned regions were adjusted manually to produce a parsimonious alignment (i.e., informative sites minimized). Phylogenetic analyses were carried out with both the entire matrix (2422 bp) and with a subset that excluded ambiguously aligned regions (65 bp deleted). The MP, ML, and Bayesian analyses of the subset matrix resulted in the same topologies and similar support to those from the entire matrix; bootstrap values for supraspecific clades in the MP analysis were the same except for three clades (1% lower in two, 4% higher in one). All results reported hereafter are those derived from analyses of the entire matrix.

Maximum parsimony (MP) and maximum likelihood (ML) analyses were performed with PAUP* 4.0b10 (Swofford, 2002). For the MP analyses, trees were found with the heuristic search algorithm using tree bisection-reconnection algorithm branch swapping. One thousand replicate searches, starting from a random tree, were carried out. Characters were unordered, equally weighted and optimized with accelerated character transformation (ACCTRAN). Clade support was evaluated with nonparametric bootstrapping (Felsenstein, 1985) with heuristic searches (1000 pseudoreplicates, with 10 random addition-sequence replicates each). Patristic distances (i.e., total length of branches between each pair of taxa in the tree) were obtained from the MP tree in PAUP*.

For the ML analyses, Modeltest v. 3.5 (Posada and Crandall, 1998) was used to find the best model of character evolution of the data. To find the ML tree, I performed iterated searches. On each iteration, model parameters were set to estimates from the previous iteration, the starting tree was found via stepwise addition and latter rearranged by branch-swapping with the tree bisection-reconnection algorithm. Parameters for the first iteration were estimated from the most parsimonious tree with the best likelihood score. Iterations were continued until additional iterations yielded identical trees. This searching procedure (often termed "successive-approximations") has been demonstrated to be as reliable as full-optimization ML searches to find optimal trees (Sullivan et al., 2005).

Bayesian analyses were conducted using pMrBayes 3.0b4 (Ronquist and Huelsenbeck, 2003; Altekar et al., 2004) on a Macintosh G5 using two parallel processors, enabled by Pooch 1.5.5 (daugerresearch.com, Dauger Research, Inc.). Six Markov chains were utilized in three analyses, the temperature parameter was set at 0.08 for Analysis 1 and 0.15 for Analyses 2 and 3, the prior for the rate matrix was a uniform dirichlet and all topologies were equally probable a priori. Analysis 1 ran for 3×10^6 generations and Analyses 2 and 3 for 1.5×10^6 generations. For each analysis, the chain was sampled every 10^3 generations. The exchange rate among adjacent chains varied from 0.68–0.74 for Analysis 1 and 0.44–0.61 for Analyses 2 and 3, indicating well-mixed chains. Convergence was determined by examining plots of parameters (likelihood score, six rate variables, four nucleotide frequencies, shape parameter of the gamma distribution, and proportion of invariant sites) against generation number. The first one

third of sampled trees were discarded as the burn-in and the remaining trees were used for estimating Bayesian posterior probabilities by a majority-rule consensus procedure in PAUP*.

Although useful in Bayesian phylogenetic analysis, a mixed model was not used for the 12S and 16S genes because the same model (GTR + *G* + I) was estimated for each gene and separate analyses of 12S and 16S yielded the same tree.

Testing previous hypotheses. *I* conducted parametric bootstrap tests (Huelsenbeck and Hillis, 1996) to evaluate whether my dataset can statistically reject a given topological hypothesis. Specifically, I tested the topology that combines all species of *Engystomops* except *E. pustulosus* in a clade (e.g., Fig. 3.1A, 1C). To apply the test we: (1) introduced a topological constraint to find the shortest tree compatible with the constrained topology (null hypothesis); (2) estimated ML parameters of a model of evolution from the observed dataset and the best constrained tree; (3) simulated 1000 replicate datasets (using Seq-Gen v.1.3.2; Rambaut and Grassly, 1997) based on the model parameters from step 3 and the best constrained tree; (4) found the best MP tree and the best MP constrained tree for each replicate dataset and calculated the difference in steps between both trees; (5) compared the observed difference in steps (best tree vs. constrained tree from observed data) with the (null) distribution of the differences obtained in step 4; I rejected the null hypothesis if the observed value was > 95% of the values from the null distribution.

3.3 RESULTS

Phylogenetic relationships. The MP strict consensus , ML tree, and Bayesian majority-rule consensus resulted in fully compatible topologies except for the placement of populations of *E. pustulosus* (Fig. 3.3). The MP analysis of 2422 characters (900 variable, 755 parsimony-informative) yielded four most parsimonious trees of length 2645 (CI = 0.499, RI = 0.818; Fig. 3.3). The log-likelihood scores of the parsimony trees ranged from –15540.99 to –15538.83 (only 2.5–4.7 log-likelihood units from the score of the ML tree). The four most parsimonious trees differed in their placement of *E. coloradorum* (either as sister taxon to *E. guayaco* or to the clade (*E. montubio* + *E. randi*)) and the intraspecific placement of two *E. pustulosus* populations from eastern Central America.

According to the Akaike information criterion (Akaike, 1974), the model with the best fit is GTR + *G* + I. Maximum likelihood analysis under that model resulted in a tree with ln *L* = –15536.32 (Fig. 3.3; shape parameter with four discrete rate categories = 0.65348; proportion of invariable sites = 0.429389; estimated nucleotide frequencies: A = 0.36420, C = 0.17661, G = 0.16541, T = 0.29378). The ML tree and the Bayesian consensus place *E. coloradorum* as the sister species of *E. guayaco*. However, the clade (*E. coloradorum* + *E. guayaco*) lacks strong support (Bayesian posterior probability = 0.92). All other supraspecific clades are well supported (Fig. 3.3). The Bayesian tree topologies for all three analyses were identical. Of the 40 internal nodes, only 7 had posterior probabilities less than 1.0 and the posterior probabilities differed by 0.02 at one node and 0.01 in the other six, indicating adequate convergence of the Markov chains.

Two allopatric basal clades are defined within *Engystomops*: one contains all species distributed in the lowlands (up to 1000 m of altitude) west of the Andes in Ecuador and northern Peru; the other contains *E. pustulosus* (Central America and northern South America) and the Amazonian *E. petersi* and *E.* cf. *freibergi* (Figs. 3.3–3.5). Within the W Ecuador-Peru clade, there is a clade of small sized species (mean male snout-vent length < 23 mm) that includes *E. coloradorum* and the recently described *E. guayaco*, *E. montubio*, and *E. randi*.

All analyses show two well supported basal clades among the 15 samples of *E. pustulosus*: (1) includes all the samples from the western range of the species (i.e., Costa Rica, Nicaragua, El Salvador and Mexico; 10–15 in Fig. 3.2); (2) includes samples from the eastern range (Panama, Colombia, and Venezuela; 1–9). Patristic distances between both clades ranged from 32 to 44 (uncorrected $p = 0.031–0.043$); patristic distances within clades ranged from 3 to 36 (uncorrected $p = 0.005–0.031$). A genetic divide among populations is evident in central Costa Rica. For example, Liberia (W Costa Rica) and Laguna Verde (SE Mexico) belong to the same clade, have a patristic distance of 9 (uncorrected $p = 0.015$), and are 1600 km apart; Liberia and Armuelles (W Panama) belong to different clades, have a patristic distance of 34 (uncorrected $p = 0.023$) even though they are separated by only 391 km (Fig. 3.2).

An additional species showing well-supported allopatric clades is *E. randi*. These clades are separated by a narrow stretch of lowland east of Golfo de Guayaquil in Ecuador (Fig. 3.5). Distances between the northern and southern clade of *E. randi* range from 10 to 14 (uncorrected $p = 0.020–0.027$).

Patristic distances (MP) ranged from 1 (between both populations of *E. coloradorum*; uncorrected *p* distance < 0.001) to 292 (between *E. enesefae* and *E. pustulosus* from Carupano, Venezuela; uncorrected $p = 0.178$). The minimum patristic distance between two unambiguously recognized species is 20 (*E. montubio* and *E. randi*; uncorrected $p = 0.029$).; those between *E.* cf. *freibergi* (Alto Juruá, Brazil) and *E. petersi* from eastern Ecuador range from 29 to 33 (uncorrected $p = 0.039–0.041$).

We present a phylogenetic classification that provides unranked names for well-supported clades within *Engystomops* (Supplemental Data 3.1; Fig. 3.6). The hierarchical position of each name is denoted by its indentation in Supplemental Data 3.1.

Testing previous hypotheses. The only major incongruence with previous phylogenies is seen in those derived exclusively or mainly from sequences of 12S rRNA and COI genes. They show *E. petersi* as sister taxon to the clade Duovox rather than to *E. pustulosus* (Ryan and Rand, 1993; Cannatella et al., 1998; Weigt et al., 2005). The parametric bootstrap test for monophyly of (*E. petersi* + Duovox) shows that this null hypothesis can be rejected with high confidence ($p = 0.004$; Fig. 3.7).

3.4 DISCUSSION

Systematics. *my* phylogeny, including all known species of *Engystomops*, is consistent with most previous systematic reviews (e.g., Cannatella and Duellman, 1984; Cannatella et al., 1998). Clades recovered previously on the basis of morphological (Cannatella and Duellman, 1984; Cannatella et al., 1998) and molecular datasets have high support in my phylogeny (Cannatella et al., 1998; Ron et al., 2005). The only exception is that my data

reject the hypothesis that *E. pustulosus* is the sister taxon to a clade comprising the remaining species of *Engystomops* (Figs. 3.1 and 3.7).

Monophyly of *Engystomops* is well supported by my analysis and the inclusion of mtDNA sequences from additional species of *Physalaemus* does not alter that outcome (DCC, unpublished data; Tarano and Ryan, 2002). As far as I know, *Engystomops* monophyly has not been questioned since it was first proposed by Lynch (1970). Therefore, the resurrection of the genus *Engystomops* as proposed by Nascimento et al. (2005) is logically consistent with the phylogeny. Although the taxonomic change was not a strict requirement of the phylogeny, the increase in the informativeness of the classification is desirable considering the large species content of the former "*Physalaemus*" (almost 50 species).

Clade Edentulus. Edentulus is distributed in Central America, northern South America, and the Amazon Basin (Fig. 3.4) and is allopatric to its sister clade, Duovox. The clade is supported by at least three morphological synapomorphies, including the absence of teeth (Cannatella et al., 1998).

Our analysis shows two well-supported allopatric clades within *E. pustulosus*. Each occupies one of two disjunct portions of the distribution range of *E. pustulosus*. The disjunction is 175 km in length, in central Costa Rica, between Barranca and Puerto Cortés (both in Puntarenas Province; Savage, 2002; Fig. 3.4). The western clade is distributed from southern Mexico to western Costa Rica; the eastern clade ranges from eastern Costa Rica to northern Colombia and Venezuela (Fig. 3.4; Weigt et al., 2005).

My results are consistent with evidence of genetic distinctiveness between both ranges in allozymes and CO I sequences (Ryan et al., 1996; Weigt et al., 2005).

Several lines of evidence suggest that *E. pustulosus* is composed of at least two cryptic species. The high support for both clades (bootstrap 99 and 100) and their concordance with geography (i.e., allopatric, with an intervening barrier to gene flow) indicate that each basal clade represents a separate species, according to the criteria of Wiens and Penkrot (2002). The use of mtDNA for species delimitation is controversial because its uniparental inheritance does not encapsulate the complete organismal history (but see Wiens and Penkrot, 2002). However, nuclear markers have uncovered the same two genetic clusters (i.e., eastern and western separated by a distributional gap in central Costa Rica; Weigt et al., 2005) suggesting that the divergence between both clades is not an artifact of differential gene flow and dispersal in females or a mismatch between the mtDNA tree and the population histories.

Species status for each clade also is suggested by a putative long period of divergence between both clades of 6 to 10 Ma (Weigt et al., 2005) and by patristic distances higher than those reported between uncontroversial sister species in Duovox (*E. montubio* and *E. randi*). Although previously overlooked, there is at least some level of morphological differentiation as well. A reanalysis of datasets (Freeman, 1967 and Cannatella and Duellman, 1984) of average body size from 34 populations and 669 individuals shows significant differences between both ranges (western populations are smaller; ANOVA $p < 0.001$, df = 33).

Ryan et al. (1996) and Weigt et al. (2005) have asserted that the allozyme differentiation between both ranges is within the limits of inter-population variation. That interpretation has been questioned (Wynn and Heyer, 2001) and is not readily supported by the observed pattern of genetic differentiation. If each clade is granted species status, the binomial *E. pustulosus* should be applied to the eastern lineage (type locality for *E. pustulosus* is "New Grenada, on the River Truando" in Colombia; Cope, 1864 in Cannatella and Duellman, 1984). No binomial is available for the western lineage and therefore the species awaits description.

Subsequent to Lynch's (1970) review, most Amazonian *Engystomops* have been assigned to *E. petersi*. The use of *E. freibergi* has been restricted to the type locality "Río Runerrabaque [= Rurrenabaque], Río Beni, Bolivia" and a few localities in SE Peru (Fig. 3.4). Cannatella and Duellman (1984) placed *E. freibergi* as a junior synonym of *E. petersi* because its diagnostic characters were dubious. However, Cannatella et al. (1998) recognized *E. freibergi* as a valid name for Southern Peruvian populations based on genetic distances, differences in male advertisement calls, and geographic proximity to the type-locality of *E. freibergi*; specimens and sequences from the type locality were not available. Similarly, my tentative assignment of the Alto Juruá population to *E. freibergi* has been based exclusively on geographic proximity to *E. freibergi*'s type locality (Fig. 3.4) and high levels of genetic differentiation relative to populations near the type locality of *E. petersi*, in Amazonian Ecuador. Differentiation in male advertisement calls can be indicative of prezygotic reproductive isolation in anurans (Gerhardt and Huber, 2002) and is extensive among some populations of Amazonian *Engystomops*. Calls from the single

southern population sampled by Cannatella et al. (1998; from Tambopata, Peru) were known to have an additional high frequency suffix (absent in the single northern population sampled, in Ecuador). Additional data has shown that the high frequency suffix is also present in *E. petersi* populations from Ecuador (see below) and therefore is not suitable to diagnose *E. freibergi.*

Regardless of nomenclature, available information suggests that Amazonian *Engystomops* are a species complex. My samples include five populations from NW Amazonia and one population in Alto Juruá, Acre, Brazil (Fig. 3.2). The NW Amazonian populations form one clade with high bootstrap support (100), sister to the Alto Juruá population. The large genetic differentiation between the NW Amazonian clade and Alto Juruá (patristic distances 29–33) suggests that each represents a separate species. Cytological studies have shown highly divergent chromosomal morphology and C-banding patterns among specimens from a single locality in Acre, Brazil, indicating the co-occurrence of two species (Lourenço et al., 1999). A conspicuous difference among populations is the addition of a high-frequency suffix to the call. The suffix is present in Yasuní (Ecuador; Fig. 3.2) and Tambopata (Peru; Fig. 3.4) but absent in Cando, Ishquiñambi, La Selva, and Puyo (Ecuador; Fig. 3.2; Boul and Ryan, 2004; SRR, unpublished). Significant inter-population differences also are evident in fundamental frequency (e.g., La Selva vs. Yasuní) and duration of the call (e.g., Yasuní vs. Tambopata; Boul, 2003). *Engystomops pustulosus* also can add a high frequency suffix to their calls but this capacity seems to be present in all populations (Ryan et al., 1996).

The distribution of Amazonian *Engystomops* extends over more than 3 million km^2 and has been sparsely sampled (Fig. 3.4). Although the populations included in my phylogeny represent a small portion of the distribution, they show considerable genetic divergence and call differentiation. On that basis, I predict that analyses encompassing a larger geographic area will reveal the existence of even more distinct lineages with complex patterns of call variation and distribution. A comprehensive analysis of the phylogeny and phylogeography of Amazonian *Engystomops* is currently underway (W. C. Funk, pers. comm.)

Clade Duovox. Despite its considerable species diversity, Duovox has a relatively restricted distribution (lowlands of western Ecuador and NW Peru; Fig. 3.5). Habitat types range from tropical deciduous dry forest to tropical evergreen moist forest. All species in this clade are locally abundant in human-disturbed regions and it seems unlikely that the widespread conversion of natural vegetation into agricultural lands has had a negative impact on their populations (Ron et al. 2005).

Duovox monophyly is strongly supported by my molecular data and morphological characters (Cannatella et al., 1998; Ron et al., 2005). Morphological synapomorphies for the group are the absence of tarsal tubercle, and a narrow stalk of the alary process of the hyoid (Cannatella et al., 1998; Ron et al., 2005).

Until 2004, only two species of Duovox had been described, *E. pustulatus* and *E. coloradorum*. Traditionally, all Duovox lacking autapomorphies of *E. coloradorum* have been assigned to *E. pustulatus*. Incorrect assignment to *E. pustulatus* might be a

consequence the brief species description based on a single juvenile specimen (Ron et al., 2004).

Engystomops pustulatus is a large species compared to its congeners (male SVL 25.17–29.88). It has a distribution restricted to western Ecuador (Fig. 3.5; Ron et al., 2004; Ron et al., 2005). Although with a conspicuously different advertisement call and external morphology, *Engystomops randi* has been incorrectly referred to *E.* "*pustulatus*" in most publications (e.g., Cannatella and Duellman, 1984; Ryan and Rand, 1993; Ryan and Rand, 1995; Cannatella et al., 1998). Comparison of the type material of *E. pustulatus* with the newly collected series enabled us to detect this misidentification.

Specimens morphologically similar to *E. pustulatus* from NW Peru have been considered a distinct species (e.g., Cannatella et al., 1998; Ryan and Rand, 2001). My phylogenetic and subsequent morphological analyses (SRR, unpublished) confirm that those populations belong to an undescribed species with a distribution restricted to NW Peru (*E.* sp. B in Figs. 3.3 and 3.5). Based on its high patristic distances (47–48), I suggest that its sister taxon (*E.* sp. D in Figs. 3.3 and 3.5) is also an undescribed species, known from few localities in SW Ecuador. *Engystomops* sp. B has been mistakenly referred as *E*. "*pustulatus*" (e.g., Cannatella and Duellman, 1984; Frost, 2004; Nascimento et al., 2005; Savage, 2002).

Species of the clade Brevivox have a smaller size than other *Engystomops* (Ron et al., 2004; Ron et al., 2005). Except for the distinctive *E. coloradorum*, this group is morphologically conservative, to the extent that species identification based on external morphology is challenging (Ron et al., 2005). Moreover, reproduction in *E. guayaco*, *E.*

montubio, and *E. randi* takes place by night, during the same season, and in similar microhabitat. In regions of sympatry, this results in males from closely related species calling next to each other in reproductive aggregations. Reproductive syntopy should exert strong selective pressures favoring interspecific divergence in advertisement call and female call preference in sympatric species. The patterns of call differentiation match these expectations because the allopatric *E. guayaco* and *E. montubio* have nearly indistinguishable calls while the sympatric *E. guayaco* and *E. randi* show markedly different call rates (Fig. 3.5; Ron et al., 2005).

Engystomops randi shows a disjunct distribution with two small ranges separated by an unoccupied region 80 km long (Fig. 3.5). In my phylogeny, four populations clustered into two clades corresponding to each of the two ranges (Figs. 3.2 and 3.3). Support for each clade is high (bootstrap = 100) suggesting limited gene flow. However, small sample size, low patristic distances (10–14), and limited differentiation in advertisement call and external morphology (SRR, unpublished) are inconclusive regarding the taxonomic status of these populations. Nuclear markers will be useful to explore this question.

Impact of molecular systematics on estimates of amphibian diversity. There is an urgent need to describe the biodiversity of tropical regions because of their extreme species richness and the rapid destruction rate of their natural habitats. The amphibian fauna of the Neotropics is an example of the prominence of the tropics in biodiversity richness given that roughly one half of all recognized amphibian species lives in Central or South America (Duellman, 1999). Moreover, the number of Neotropical amphibians

that await description may be high considering that the rate of discovery of amphibians exceeds that of any other vertebrate group, and a majority of the newly described species is from tropical regions (Cannatella and Hillis, 2004). To my knowledge, no attempts have been made to estimate the number of undescribed amphibian species.

Although only a few intraspecific and species-level molecular phylogenies are available for Neotropical amphibians, the available data suggest that a significant number of species have either been overlooked by morphology-based taxonomic reviews or have not been sampled at all. A list of some of those molecular analyses, including numbers of undescribed species discovered, is shown in Table 3.2. The numbers of undescribed species are based on each study authors' explicit decisions. In two studies where the authors did not make decisions to define species limits (*Bufo marinus* and 30-chromosome *Hyla*), I applied the Wiens and Penkrot (2002) criteria to delimit species. In both cases, cryptic species were found according to criterion c (Fig. 3.1 in Wiens and Penkrot, 2002).

The data show 35 undescribed species discovered in 7 studies, a 28% increase to the 123 described species included (Table 3.2). This percentage is even higher (39%) for studies where taxon sampling has been more intensive (i.e., studies that included more than 50% of the described species). Given that there are approximately 2800 described species in the Neotropics (Amphibia Web, 2005) and assuming, conservatively, that the proportion of undescribed amphibians lies between 0.28 and 0.39, then the number of species awaiting description should lie between 784 and 1092, a figure comparable to the total described amphibian diversity of Africa.

This estimate is conservative because the proportion of discovered species should increase with taxon sampling (sampling was exhaustive only in *Engystomops* and *Sierrana*). Taxon sampling is frequently constrained by tissue availability. Therefore, studies with restricted sampling often include predominantly species of easy access, available in the pet trade and/or distributed in habitats of easy reach (e.g., poison-arrow frogs, genus *Dendrobates*) that, because of their accessibility and conspicuousness, are already described. Additionally, many groups included in Table 3.2 are composed primarily of "weedy" species, common in human-disturbed areas and therefore more likely present in scientific collections. Molecular phylogenies of species occurring in forested habitats that are difficult to access (e.g., some *Eleutherodactylus* and centrolenids) are likely to include larger proportions of unknown species.

Table 3.1. Localities, voucher information, and GenBank accession numbers for the specimens and sequences used in the phylogenetic analyses. Locality numbers correspond to those in Fig. 3.2.

Species	Locality no.[1]	Locality	Catalog or sample no.[2]	Accession no.
P. nattereri	--	Brazil: São Paulo: Luis Antônio	ACJ 95.267	DQ337208
P. signifer	--	Brazil: Rio de Janeiro: Seropédica, Hôrto Florestal Santa Cruz	TNHC 60073	DQ337209
P. albonotatus	--	Argentina: Chaco: San Fernando, 4.5 km SE Resistencia, S 27° 25' 55.6"; W 58° 54' 46.7"	DCC-NB 019	DQ337210
P. enesefae	--	Venezuela: Guárico: Calabozo	MR 005	DQ337211
P. biligonigerus	--	Argentina: Salta: Finca San Ramón, 1 km N La Merced, S 24° 57' 8.6", W 65° 29' 32.5"	DCC-NB 012	DQ337212
P. barrioi	--	Brazil: São Paulo: Parque Nacional Serra do Bocaina, 1 km before entrance	TNHC 60105	DQ337213

Species	Locality no.[1]	Locality	Catalog or sample no.[2]	Accession no.
E. pustulatus	16	Ecuador: Los Ríos: Patricia Pilar	QCAZ 19606	DQ337214
E. pustulatus	17	Ecuador: Guayas: Cerro Blanco	QCAZ 23420	DQ337215
E. sp. B	18	Peru: Lambayeque: Olmos, 8.5 km N Motupe	MR 726	DQ337216
E. sp. D	19	Ecuador: El Oro: Puyango	QCAZ 26981	DQ337217
E. sp. D	20	Ecuador: Loja: Zapotillo	QCAZ 26959	DQ337218
E. guayaco	21	Ecuador: Guayas: 11km N Cerro Masvale	QCAZ 23533	DQ337219
E. guayaco	22	Ecuador: Guayas: Naranjal	QCAZ 23652	DQ337220
E. coloradorum	23	Ecuador: Pichincha: road between Santo Domingo and Alluriquín	QCAZ 19294	DQ337221
E. coloradorum	24	Ecuador: Pichincha: 4 km NW La Florida	QCAZ 19418	DQ337222
E. montubio	25	Ecuador: Guayas: Balsas	QCAZ 23323	DQ337223
E. montubio	26	Ecuador: Manabí: Pedernales	QCAZ 23199	DQ337224

Species	Locality no.[1]	Locality	Catalog or sample no.[2]	Accession no.
E. randi	19	Ecuador: El Oro: Puyango	QCAZ 23768	DQ337225
E. randi	17	Ecuador: Guayas: Cerro Blanco	QCAZ 23425	DQ337228
E. randi	30	Ecuador: Guayas: Cerro Masvale	QCAZ 19559	DQ337227
E. randi	29	Ecuador: El Oro: Pasaje	QCAZ 19602	DQ337226
E. cf. *freibergi*	31	Brazil: Acre: Reserva Extrativista do Alto Juruá, Tejo River	ZUEC 9511	DQ337229
E. petersi	32	Ecuador: Pastaza: El Puyo	QCAZ 26210	DQ337230
E. petersi	33	Ecuador: Napo: Cando	QCAZ 11965	DQ337231
E. petersi	34	Ecuador: Napo: Napo-Galeras, Ishquiñambi	QCAZ 14723	DQ337232
E. petersi	35	Ecuador: Orellana: Estación Científica Yasuní, Universidad Católica del Ecuador	QCAZ 12128	DQ337233
E. petersi	36	Ecuador: Sucumbíos: La Selva	QCAZ 23976	DQ337234

Species	Locality no.[1]	Locality	Catalog or sample no.[2]	Accession no.
E. pustulosus	1	Venezuela: Guárico: Calabozo	LLV4	DQ337235
E. pustulosus	2	Venezuela: Sucre: Carupano	CARU	DQ337236
E. pustulosus	3	Colombia: Tolima: Mariquita	COL1	DQ337237
E. pustulosus	4	Venezuela: Zulia: Maracaibo Lake, 2.5 km from Santa Elena	SEV1	DQ337238
E. pustulosus	5	Panama: Panama: Kent's Marsh Gamboa	KM91	DQ337239
E. pustulosus	6	Panama: Veraguas: Santiago	SANT1-PAN	DQ337240
E. pustulosus	7	Panama: Chiriquí: Puerto Armuelles	PA2-PAN	DQ337241
E. pustulosus	8	Panama: San Blas: Nusagandi	NUS1-PAN	DQ337242
E. pustulosus	9	Panama: Panama: Perlas Islands	REY1-PAN	DQ337243
E. pustulosus	10	Costa Rica: Guanacaste: Liberia	LW96	DQ337244
E. pustulosus	11	Nicaragua: Managua: Managua	LW1083	DQ337245

Species	Locality no.[1]	Locality	Catalog or sample no.[2]	Accession no.
E. pustulosus	12	El Salvador: San Miguel	LW1067	DQ337246
E. pustulosus	13	Mexico: Chiapas: Puerto Madera	LW1033	DQ337247
E. pustulosus	14	Mexico: Chiapas: Tehuantepec	LW1022	DQ337248
E. pustulosus	15	Mexico: Veracruz: Laguna Verde	LW101A	DQ337249

[1] Numbers correspond to those in Figs. 2 and 3.

[2] Abbreviations: ACJ: A. Cardoso field no.; DCC: D. C. Cannatella field no.; MJ: M. J. Ryan field no.; QCAZ: Museo de Zoología Pontificia Universidad Católica del Ecuador; DCC-NB: N. Basso field no.; TNHC: Texas Memorial Museum; ZUEC: Museu de História Natural, Universidade Estadual de Campinas. Specimens for *E. pustulosus* samples were not preserved and its catalog numbers are L. W. Weigt vial codes.

Table 3.2. Numbers of described and undescribed species of Neotropical amphibians in molecular systematics studies. Quantity (a) refers to the number of extant described species when the study was performed; (b) refers to the number included in the study. Note that there is a large proportion of undescribed species discovered. Figures were extracted from the ingroup taxa only. To be conservative, the species listed as discovered in *Engystomops* only include *E. montubio*, *E. randi*, *E. guayaco*, and *E*. sp. B.

	Described species included (b)	Total no. of described species in target group (a)	Proportion sampled (b/a)	Undescribed species discovered	Increase %	Source
2. 1. *Bufo marinus*	1	1	1.00	2	200	Slade and Moritz, 1998
3. 2. Andean *Gastrotheca* Ecuador	6	6	1.00	3	50	Duellman and Hillis, 1987
3. *Engystomops*	5	5	1.00	At least 4	80	This publication; Cannatella et al., 1998
4. 4. *Sierrana*	34	42	0.81	8	23	Hillis and Wilcox, 2005
5. 5. 30-chromosome *Hyla*	12	38	0.32	1	8	Chek et al., 2001
6. Dendrobatidae	62	210	0.29	15	24	Santos et al., 2003; Vences et al., 2003
7. *Pseudoeurycea bellii* complex	3	3	1.00	2	67	Parra-Olea et al., 2005
6. Subtotal for b/a > 0.5	49	57	0.86	19	39	--
7. (studies 1 to 4 in this column)						
8. Overall total	123	305	0.40	35	28	--

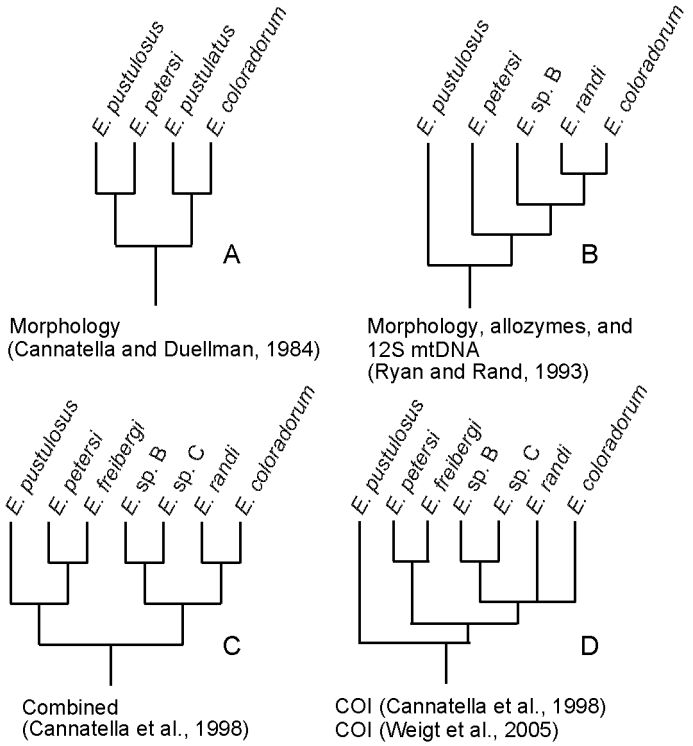

Figure 3.1. Previous phylogenetic hypotheses for *Engystomops*.

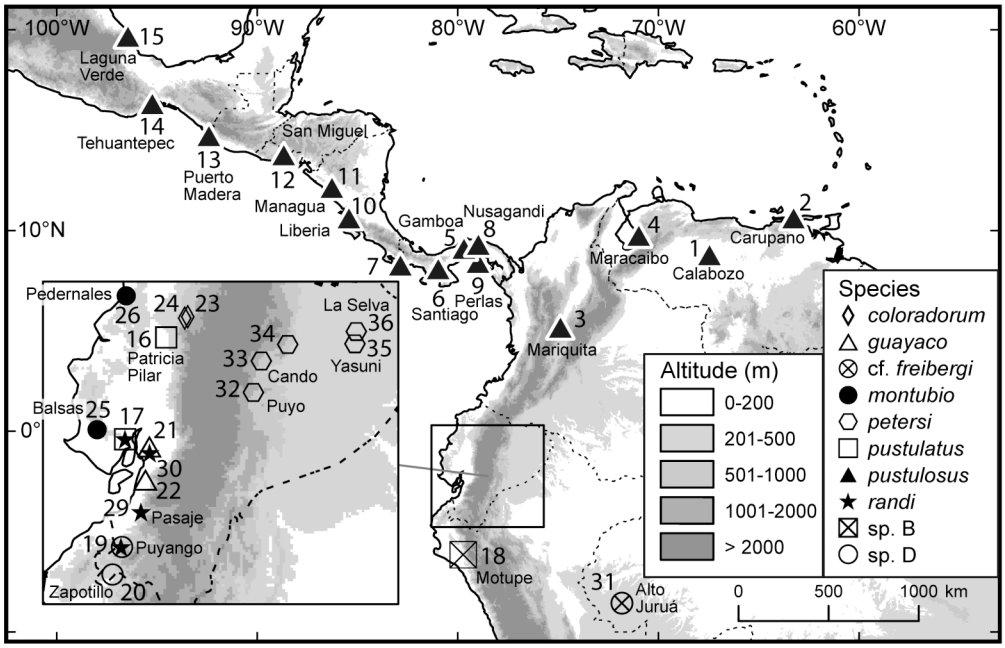

Figure 3.2. *Engystomops* sampled in the study. Locality numbers correspond to those in Figure 3.3 and Table 3.1.

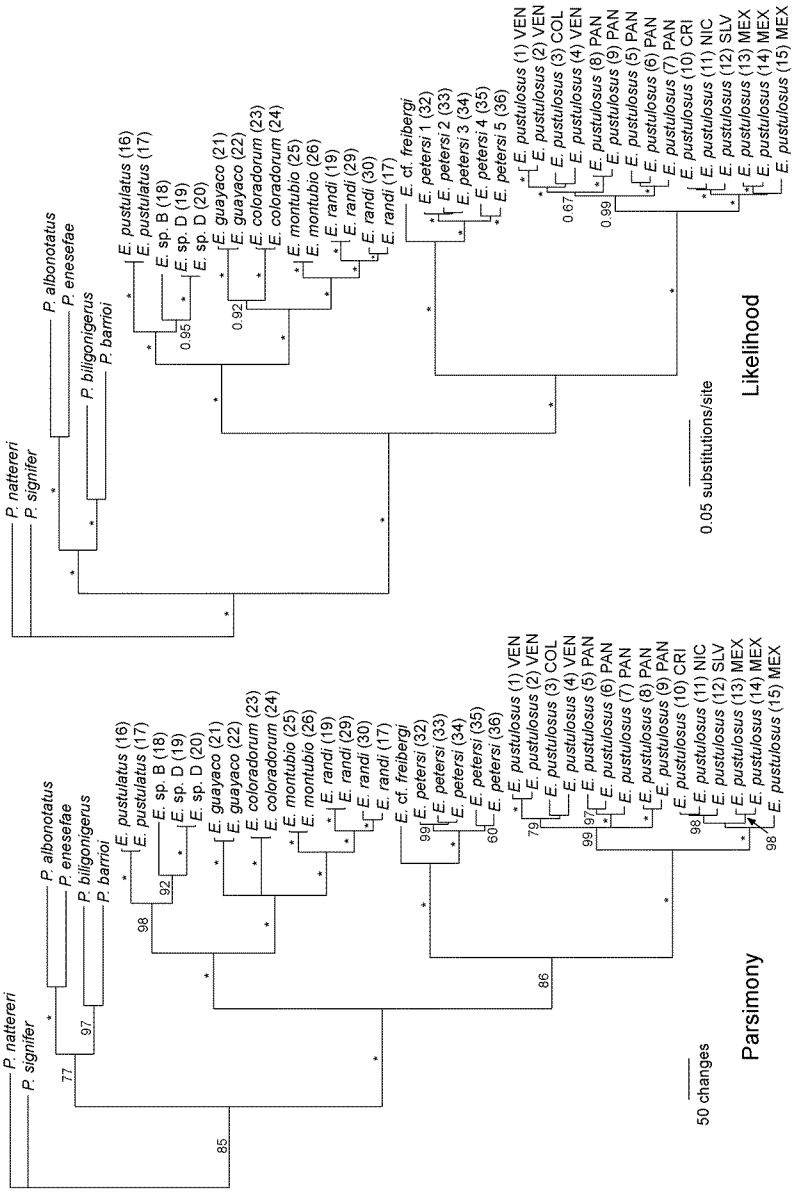

Figure 3.3. Phylogenetic relationships for members of *Engystomops*. Left: strict consensus from four most parsimonious trees (TL = 2645 steps; 755 parsimony-informative characters; CI = 0.499, RI = 0.818). Numbers at branches are nonparametric bootstrap values (from 1000 pseudoreplicates); asterisks represent 100 values. Right: maximum likelihood tree (ln L = –15536.32) obtained under the GTR + Γ + I model of character evolution. Numbers on branches are Bayesian posterior probabilities; asterisks represent 1.0 values. Abbreviations are COL: Colombia; CRI: Costa Rica; MEX: Mexico; NIC: Nicaragua; PAN: Panama; SLV: El Salvador; VEN: Venezuela. Numbers in parentheses correspond to those of localities in Figure 3.2 and Table 3.1.

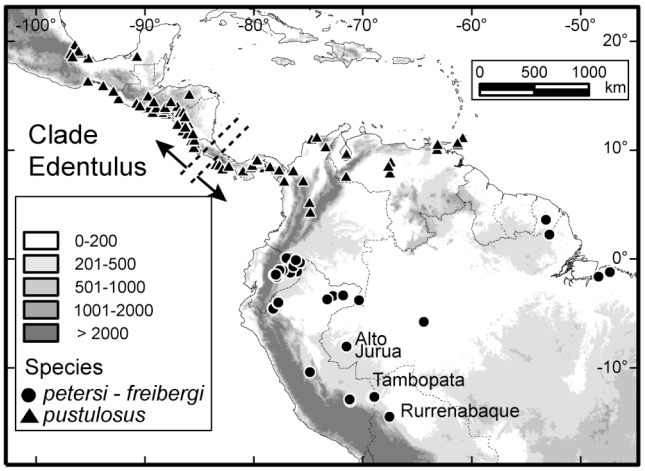

Figure 3.4. Distribution of the clade Edentulus based on museum and literature records (Cannatella and Duellman, 1984; California Academy of Sciences; Museo de Zoología Pontificia Universidad Católica del Ecuador; Museum of Comparative Zoology Harvard University, Museum Michigan State University, Natural History Museum University of Kansas, Museum of Vertebrate Zoology University of California Berkeley, and Texas Cooperative Wildlife Collection Texas A&M University). The dashed lines in Central America demarcate the gap in the distribution of *Engystomops pustulosus*. Populations on each side of this gap fall into each of two genetically divergent clades.

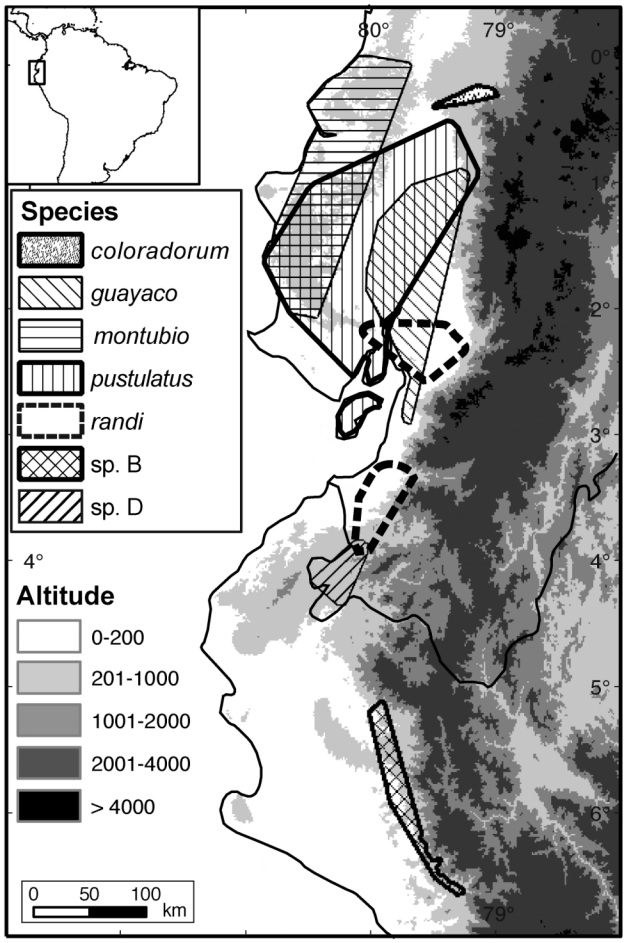

Figure 3.5. Distribution polygons of Duovox based on museum records and the literature (Ron et al. 2004; Ron et al. 2005; Museo de Historia Natural Universidad Nacional Mayor de San Marcos, Museum of Vertebrate Zoology University of California Berkeley).

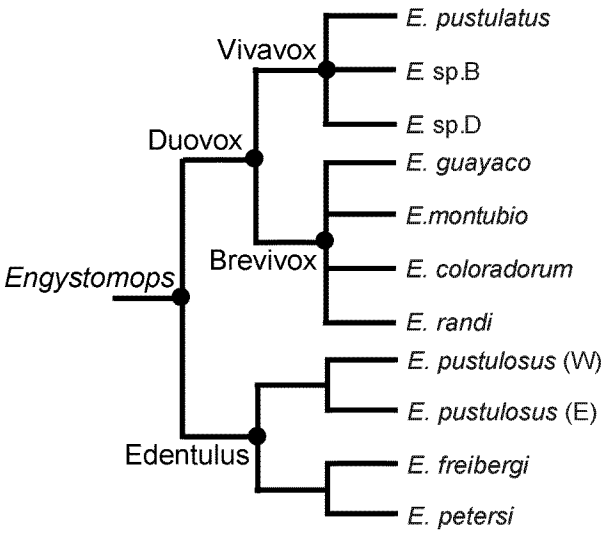

Figure 3.6. Phylogenetic classification for *Engystomops*.

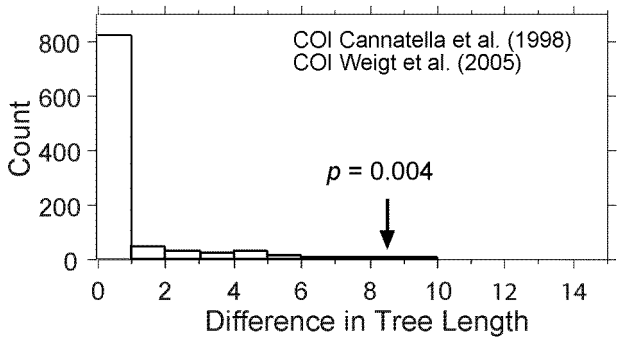

Figure 3.7. Parametric bootstrap test for monophyly of *E. petersi* + Duovox (H_0). The graph shows the difference in steps (parsimony) between the null and test hypothesis for 1000 replicates. The observed difference (arrow) was > 99.5% of the values from the null distribution (H_0 rejected).

Supplemental Data 3.1. Phylogenetic classification of *Engystomops*.

I. *Engystomops*, Jiménez de la Espada 1872 (converted clade name). Definition: clade stemming from the most recent common ancestor of *E. petersi* Jiménez de la Espada 1872, and *E. pustulatus* (Shreve, 1941). Content: all species in the definition, as well as *E. freibergi* (Donoso-Barros, 1969), and all species in Duovox (see below). Type species: *E. petersi* Jiménez de la Espada 1872. Comments: Lynch (1970) stated that *P. moreirae* may belong to *Engystomops* (= "*P. pustulosus* group"). Haddad and Pombal (1998) and Nascimento et al. (2005) included *P. moreirae* in the *P. signifer* species group.

A. Duovox, new clade name. Definition: clade stemming from the most recent common ancestor of *E. pustulatus* (Shreve, 1941), and *E. randi* (Ron, Cannatella, and Coloma, 2004). Etymology: from the Latin *duo*, meaning "duet" and *vox*, meaning "voice", in reference to their breeding aggregations that often include males from two species from this clade, calling next to each other. Content: species in the definition, as well as *E. coloradorum* (Cannatella and Duellman, 1984), *E. guayaco* (Ron, Coloma, and Cannatella, 2005), *E. montubio* (Ron, Cannatella, and Coloma, 2004), and two undescribed species from SW Ecuador and NW Peru (*E.* sp. B and *E.* sp. D in Fig. 3.3).

1. Brevivox, new clade name. Definition: clade stemming from the most common ancestor of *E. coloradorum* (Cannatella and Duellman, 1984), *E. guayaco* (Ron, Coloma, and Cannatella, 2005), *E. montubio (*Ron, Cannatella, and Coloma, 2004), and *E.* randi (Ron, Cannatella, and Coloma, 2004). Etymology: from the Latin *brevis*, meaning "short" and *vox*, meaning "voice"; it refers to both the short

duration of their calls and their small body size compared to other members of *Engystomops*. Content: species in the definition.

2. Vivavox, new clade name. Definition: clade stemming from the most recent common ancestor of *E. pustulatus* (Shreve, 1941) and the undescribed species from Puyango, Ecuador (*E.* sp. D in Fig. 3.3). Etymology: from the Latin *viva voce*, meaning "aloud"; it refers to the loudness of their advertisement calls. Content: species in the definition plus *E.* sp. B (Fig. 3.3).

B. Edentulus, new clade name. Definition: clade stemming from the most recent common ancestor of *E. pustulosus* (Cope, 1864) and *E. petersi* Jiménez de la Espada 1872. Etymology: from the Latin *edentulus*, meaning "toothless"; it refers to the absence of maxillary and premaxillary teeth, a synapomorphy for the clade (Cannatella et al., 1998). Content: species in the definition, as well as *E. freibergi* (Donoso-Barros, 1969).

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Chapter 4

Testing the paradigm of sexual selection by sensory exploitation

Abstract. Female preference can favor the evolution of male secondary sex traits that are maladaptive under natural selection. A mechanism increasingly explored to explain the origin of such male traits is sensory exploitation. Under sensory exploitation, female mating preferences are by-products of sensory biases that originated previously in other contexts. Túngara frogs offer one of the most widely known examples for this mechanism. Male túngara frogs make a complex call by adding a chuck to their typical whine call. Previous studies reported that the evolution of the complex call, which is preferred by females, was driven by a pre-existing female preference. Here, I reevaluate the sensory exploitation hypothesis using a comprehensive phylogeny and increased taxon sampling of both the female preference and the male trait. My results do not support an early origin of the preference prior to the origin of the complex call, as predicted by sensory exploitation, but rather demonstrate that the preference for complex calls originated only recently. Mate choice experiments demonstrate that increased stimulation of the sensory system does not necessarily translate into increased preference, highlighting the significance of central processing in mate choice decisions. Maximum likelihood tests support models of sexual selection that predict coevolution between male trait and female preference because they revealed a significant phylogenetic correlation between them.

4.1. INTRODUCTION

Strong female preference for costly, conspicuous, or elaborate secondary sexual traits in males explains why such seemingly disadvantageous traits are maintained. How such traits are initially favored, however, is an on-going debate. The sensory exploitation hypothesis (also known as "sensory bias") states that female mating preferences are a byproduct of pre-existing sensory biases, which evolved incidentally, under other selective pressures (e.g., prey acquisition). Subsequently, males evolve secondary sexual characters that match those biases (e.g., match the color of prey) because they are preferred by females (West-Eberhard, 1984; Ryan and Rand, 1990; Ryan and Rand, 1993b; Fuller et al., 2005; Basolo, 1995). Unlike other sexual selection models (e.g., good genes or Fisherian selection), the sensory exploitation hypothesis requires the preference to evolve before the trait.

Túngara frogs (genus *Engystomops*, formerly known as the *Physalaemus pustulosus* species group; Nascimento et al. 2005) have been considered a textbook example of sensory exploitation (Ryan and Rand, 1993b; Ryan and Rand, 1993a; Ryan and Rand, 2003a). For reproduction, males aggregate at night to call in choruses. Females visit these choruses and actively choose a mate based on acoustic features of males' calls (Ryan, 1985). The advertisement call consists of a whine-like sound that is necessary and sufficient for mate recognition. In *E. pustulosus*, *E. freibergi*, and some populations of the *E. petersi* species complex, males can add a facultative "chuck" to the call (Ryan, 1985; Fig. 4.1A). In *E. pustulosus* and at least one population of *E. petersi*, the chuck enhances call attractiveness to females (Ryan, 1985; Boul et al., 2007). Traditionally, calls with chucks and without chucks have been referred to as "complex" and "simple",

respectively. Sensory exploitation was invoked in these species because the origin of the female preference for the chuck seemed to precede the origin of the chuck itself (i.e., the chuck exploits a preexisting sensory bias; Fig. 4.1B; Ryan and Rand, 1993b). The ancestral status of the preference was inferred from mate-choice experiments showing that females of a congener, *E. coloradorum*, prefer calls to which three chucks were artificially appended even though *E. coloradorum* males do not produce complex calls (Fig. 4.1B; Ryan and Rand, 1993b). The phylogeny therefore suggested that complex calls had originated more recently than the female preference and therefore exploited a sensory bias of prior origin (Fig. 4.1B; Ryan and Rand, 1993b).

In addition to the sensory exploitation hypothesis, there is a complementary, mechanistic hypothesis for the preference for complex calls based on neurophysiological characteristics of the frog's inner-ear (hereafter referred to as the frequency-matching hypothesis; Wilczynski et al., 2001). The acoustic sensitivity of the amphibian ear resides in two organs, the amphibian papilla (AP: low frequencies) and the basilar papilla (BP: high frequencies). The frequency of a whine-chuck call has two emphasized frequency ranges, and each overlaps with one of these organs: AP (whine) and BP (chuck). In contrast, whine-only calls have been considered to stimulate solely the AP (Ryan, 1990; Ryan et al., 1990; Wilczynski et al., 2001). The frequency-matching hypothesis states that whine-chuck calls are more attractive because, unlike whine-only calls, they also stimulate the BP. Thus, calls considered to be complex by Ryan and Rand (2003a) should have a higher content of energy stimulating BP than simple calls. Because the sensory exploitation hypothesis predicts that the attractive male trait originates after the origin of

the female preference (Fig. 4.1B), it follows that calls stimulating AP-only should be ancestral, whereas calls stimulating both the AP and BP should be recently derived.

The proposed evolutionary scenarios for sensory exploitation in túngara frogs rely on ancestral character reconstruction, which can be biased by under-sampling of the trait distribution as a result of taxon exclusion (Ackerly, 2000). This compromises the sensory exploitation hypothesis primarily because until recently (Ryan and Rand, 2003a) sampling of the female preference included only two species of túngara frogs. Although sampling for both the female preference and the male signal have increased (Ron et al., 2004; Ron et al., 2005; Tárano and Ryan, 2002; Boul et al., 2007), it is still incomplete. In addition, new species have been discovered and a comprehensive and well supported phylogeny was recently published (Ron et al., 2006).

Herein I present new female preference information and take advantage of improved understanding of the phylogeny and signal diversity in túngara frogs to reexamine the evolution of female preference and the evidence supporting the sensory exploitation hypothesis. Specifically, I test the critical prediction of the sensory exploitation hypothesis: that the female preference for the chuck (i.e., the "exploitative" signal) originated before the chuck itself. I also evaluate the evolution of calls in the context of the frequency matching hypothesis.

4.2. MATERIALS AND METHODS

Túngara frogs belong to the genus *Engystomops* (formerly known as the *Physalaemus pustulosus* species group). They form a clade of ~10 species of small (16–35 mm) frogs. This group is most closely related to *Physalaemus* (Ron et al., 2006). Species nomenclature has changed recently (Nascimento et al., 2005) and I follow the

most recent review (Ron et al., 2006). See Supplemental Data 4.1 in for equivalencies in species names among relevant publications.

Female preference and male trait. I used phonotaxis experiments (Ryan and Rand, 1990) to assess whether the chuck of *E. pustulosus* enhances attractiveness of male calls in three species of túngara frogs that do not produce chucks (*E. coloradorum*, *E. randi*, and *E.* sp. D) . Each phonotaxis experiment had at least 19 replicates. In each replicate, a wild-caught gravid female was given a choice between two male advertisement calls broadcast antiphonally from speakers at opposite ends of a rectangular arena. One call was the conspecific advertisement call whereas the other was the same call with a single chuck from *E. pustulosus* artificially appended to the end. A choice was scored when the female came within 10 cm of either speaker. Experiments were carried out between January and February 2005 in two localities in western Ecuador: Tinalandia, Provincia de Pichincha (*E. coloradorum*), and Puyango, Provincia El Oro (*E. randi*, and *E.* sp. D). At Puyango, *E.* sp. D and *E. randi* are syntopic during the reproductive season.

Females were collected between 1900 and 0100 hs from choruses; most females were in amplexus. Experiments were carried out at night, under complete darkness in a chamber (180 x 105 x 100 cm) with walls covered with padded foam to reduce reverberation. The distance between the centre of the arena and each speaker was 80 cm.

Before each trial the female was placed in the centre of the arena under a funnel. Then, two alternative stimuli were played for three minutes, after which the funnel was lifted. Female movements were monitored in real-time on a TV screen attached to an infrared video camera (Sony DCR-TVR70) located on the arena ceiling. The stimuli were broadcast either from an iBook G4 or a Toshiba Satellite 1100-s101 computer connected

to two speakers (Saul Mineroff Field Speakers SME-AFS). Each stimulus was played from a different channel of a stereo sound file and they were emitted antiphonally through the speakers. The peak amplitude of the call at the release site was 80 dB SPL (re 20 μP). The amplitude was measured with a Radioshack No. 33–2055 sound pressure level meter over a calibrated pure tone (10 sec long). The speakers faced each other from opposite ends of the chamber. Both stimuli were normalized to the same peak amplitude for the whine. The call rate per channel was 0.5 calls/sec for *E. coloradorum* and *E.* sp. D and 1.4 calls/sec for *E. randi*. To avoid biases, I randomized the side from which the stimuli were broadcast in all trials. The floor of the chamber was always wet and the temperature within the chamber was controlled.

To score female choices, I followed methodology of Ryan & Rand (2003b). For each trial, a choice was scored when the female came within 10 cm of a speaker. No choice was scored if the female either: (i) remained at her initial position for > 5 minutes, (ii) moved and then remained motionless for > 2 minutes, (iii) climbed the wall for > 2 minutes, (iv) reached the speaker by drifting along the arena edge, or (v) did not make a choice 15 minutes after the funnel was lifted.

I digitally appended a synthetic chuck to the natural conspecific advertisement call. I employed replicate stimuli with natural calls from different males from the same population. Stimuli were prepared from calls of 5 males in *E. randi*, 12 males in *E. coloradorum*, and 12 males in *E.* sp. D. Males were recorded in the field with a Sennheiser™ ME-67 directional microphone attached to an analog recorder SONY™ WM-DC6 or digital recorders SONY™ TCD-D8 or SONY™ MZ-NH1. All signals were prepared with Cool Edit pro 2.0 (© Syntrillium Software Corporation). *Engystomops*

coloradorum and *E.* sp. D can facultatively add a high-frequency suffix to the call; I did not use calls with suffixes in any test.

In each trial, the female was exposed to its unmodified conspecific call and the same call to which the chuck was appended. Thus, in each trial, each female heard calls from the same male (modified vs. unmodified). The specific replicate pair of stimuli to be used in each trial was selected with a random number generator.

For each experiment, I calculated the exact two-tailed binomial probability of the null hypothesis of no preference. Scoring of the preference as present or absent was based on the *P* value from the binomial tests (a "preference" exists if $P < 0.05$). I also report Bayesian analyses (Wilczynski et al., 1999) showing the relative probability that the observed proportion is drawn from any one of several *a priori* preference proportions (0.10, 0.25, 0.50, 0.75, and 0.90). The 0.50 proportion is equivalent to the two stimuli being equally attractive; the 0.75 proportion is equivalent to the stimuli being just different enough to be discriminable by females (Wilczynski et al., 1999).

To assess the evolutionary origin of the chuck, I scored the chuck as present or absent, following Ryan & Rand (2003a). Scoring the presence or absence of the chuck is not trivial because recent fieldwork showed that the advertisement calls of *E. coloradorum*, *E. pustulatus*, *E.* sp. B, and *E.* sp. D contain suffixes that have some structural resemblance to the chuck of *E. pustulosus*, *E. freibergi*, and *E. petersi* (Fig. 4.2). Ryan and Rand's (1993a; 2003a) scoring is based on real and salient acoustic features that are uniquely combined in the chuck. These include: (i) the fundamental harmonic (half the frequency of the fundamental harmonic in the whine), (ii) the number of harmonics (double those in the whine), (iii) a significant increase in dominant

frequency relative to the whine, and (iv) a sudden increase in call amplitude. Calls of *E. montubio*, *E. guayaco*, and *E. randi* (N), and *E.* sp. D were not available to Ryan & Rand (1993a; 2003a). I scored them as lacking the chuck because they always lack feature (i) and (iv) and almost always lack feature (ii).

Ancestral state reconstruction. To reconstruct ancestral female preferences, I also included preference data from the literature. I included data for *P. enesefae* from Tárano and Ryan (2002), *E. pustulosus* from Ryan and Rand (1990), and *E. petersi* from Boul et al. (2007) and Guerra (2005). Experiments with *E. petersi* included three populations from which only one (Yasuní) produces chucks. In the three populations, females had to choose between the local whine vs. the local whine with a chuck from Yasuní appended (Guerra, 2005; Boul et al., 2007). The conclusions from my analyses remain the same even if *E. petersi* is excluded from the analyses (results not shown).

I used maximum parsimony and maximum likelihood (ML; Pagel, 1994) to reconstruct ancestral character states for both male trait and female preference. The male trait and the female preference were coded as discrete binary characters (present-absent). Parsimony and ML reconstructions were implemented using Mesquite 1.12 (Maddison and Maddison, 2005). The ML reconstruction applied a "global" estimator under a Markov model (Pagel, 1994) with equal rates of change between states. The ML rate estimate was $\alpha = \beta = 107$ for female preference and 2.258 for male trait. Unequal rates of change (i.e., $\alpha \neq \beta$) did not fit the data significantly better (preference: LR = 1.35, ML ratio test $P > 0.05$; trait: LR = 1.12, ML ratio test $P > 0.05$). All reconstructions are based on the maximum likelihood phylogeny and branch lengths of Ron et al. (2006). The

sample of outgroup taxa for the male trait was directed to maximize representation of basal lineages within *Physalaemus*.

An additional coding strategy for maximum parsimony reconstruction of the female preference, based on the Bayesian analysis of the experimental data was also applied. Under this strategy, the preference was coded as an ordered character with 5 states (1 to 5). Each state corresponds to each of the five *a priori* preference proportions 0.10, 0.25, 0.50, 0.75, and 0.90 that were used as reference to estimate posterior probabilities. The state assigned to each species corresponds to the preference proportion with the highest posterior probability (Table 4.1). The results from this analysis (Supplemental Data 4.2) are similar to those of the binary parsimony reconstruction and are not discussed further.

To avoid information loss resulting from coding a continuous variable as a binary character, I also reconstructed ancestral female preference as a continuous character (proportion of females preferring the whine-chuck call in phonotaxis experiments) with squared-change parsimony with weighted branches (Maddison, 1991) as implemented in Mesquite 1.12 (Maddison and Maddison, 2005).

Testing the correlation between male trait and female preference. Testing for a correlation in the evolution of the sexual trait and its corresponding female preference can help to discriminate among alternative hypotheses of the underlying process of sexual selection. I applied Pagel's (1994) test for correlated evolution of two characters to test the correlation between the chuck presence and its preference in females. The test generates a null distribution of likelihood ratios between two evolutionary models (Pagel, 1994). Under the first model, the evolution of both characters is considered dependent because the rate of change between character states (e.g., preference present-absent)

varies depending on the state of the other character (e.g., chuck present-absent). The second model is similar except that characters evolve independently. The null distribution was generated from 100 simulations of characters evolving independently along the phylogeny. H_0 was rejected if the observed likelihood ratio between the two models was > 95% of the simulated ratios. All computations were carried out using the software Mesquite v. 1.12 (Maddison and Maddison, 2005).

Frequency allocation and matching. According to the frequency-matching hypothesis, whine-chuck calls are preferred because they stimulate both ear receptors (AP and BP; whine-only calls stimulate the AP only). This hypothesis predicts that calls from species with whine-chuck calls should stimulate more strongly the BP than calls from species with whine-only calls. I tested this prediction with two analyses. First, I measured the proportion of energy greater than 1.5 kHz and tested for differences, taking phylogeny into account, between complex calls and simple calls. The proportion of energy greater than 1.5 KHz quantifies the relative amount of energy available for BP stimulation (BP sensitivity is absent below 1.5 KHz in *Engystomops* and *Physalaemus*; Wilczynski et al., 2001). I chose 1.5 kHz as threshold following Rand et al. (1992) who used it to study the effect of differential stimulation of the AP and BP on female preference. The test is based on a maximum likelihood ratio from the generalized least squares (GLS) approach proposed by Pagel (1997). A significant result would indicate that the mean value of the quantitative trait (energy content) differs between complex and simple calls. Analyses were carried out with the software package Continuous (described by Pagel, 1997) under the phylogeny presented by Ron et al. (2006).

The first test does not compare the tuning of the ear between simple and complex calls. In a second test, I compared the frequency-tuning match in six species (five with simple and one with complex calls), between the upper frequency peak of the male call (calls have two peaks; Fig. 3) and the best excitatory frequency of the BP. Raw measurements for best excitatory frequencies were provided by W. Wilczynski and are summarized in Wilczynski et al. (2001). Comparisons were carried out with Kruskal-Wallis tests.

Call energy content and peak frequency were measured using CANARY 1.2.1 (Charif et al., 1995). Voucher and locality information for call recordings are listed in Supplemental Data 4.3. All recordings were made in the field except for one *E. coloradorum* recorded in captivity a few hours after capture.

In *E. coloradorum*, *E. pustulatus*, *E.* sp. B, and *E.* sp. D, I only selected calls containing high frequency suffixes (to be consistent with the choice of chuck-appended calls in *E. pustulosus* and *E. petersi*). *Engystomops freibergi* from Tambopata are known to produce chuck-appended calls (Fig. 4.4). However, calls with chucks from Tambopata were not available for analysis. The calls analyzed are from a population with whine-only calls. All *E. pustulosus* calls had one chuck.

If several calls were available from each individual's recording, I selected a single call with a random number generator. For each terminal taxon, I analyzed calls from five individuals using the software CANARY 1.2.1 (Charif et al., 1995). Each call was filtered above and below its call's frequency range to decrease background noise (usually below 0.15 KHz and above 6 KHz). Relative energy content (as energy flux density) was

measured over a spectrogram generated with a fast Fourier transformation with a frequency resolution of 87.4 Hz and 2048 points (sampling frequency = 44.1 KHz).

Because the sensory exploitation hypothesis predicts that the complex male trait is recently derived (Fig. 4.1B), calls stimulating both the AP and BP must also be recently derived (AP-only calls are ancestral). Thus, the most recent common ancestor (MRA) of *Engystomops* should have had little energy available for BP stimulation. I explored this prediction by reconstructing the proportion of energy > 1.5 KHz using squared-change parsimony with weighted branches (Maddison, 1991), as implemented in Mesquite 1.06 (Maddison and Maddison, 2005).

4.3. RESULTS

Female preference and Male trait. Females of the three species tested demonstrated the ability to make phonotactic discriminations during the experiments by showing strong preference for their natural call versus white noise (Table 4.2).

Chuck preference tests indicate that, contrary to predictions of the sensory exploitation hypothesis, addition of the *E. pustulosus* chuck to the whine does not increase call attractiveness in any of the three tested species (Fig. 4.5; Table 4.1). In fact, adding the chuck decreased female preference in *E. randi* (Fig. 4.5). In *E. coloradorum*, results show lack of preference and are inconsistent with Ryan & Rand's (1993b) finding of an increased preference for calls with chucks (see *Discussion*).

Chuck preference tests have been carried out in 8 taxa (this paper and in Tárano and Ryan, 2002; Ryan and Rand, 1990; Boul et al., 2007; Guerra, 2005). Females have shown a significant preference for the chuck in only two of them, precisely in those species that produce chucks.

The present reconstruction of ancestral female preference (Fig. 4.6) is inconsistent with the sensory exploitation hypothesis because it indicates that the preference for the chuck has evolved recently in the lineage leading to *E. pustulosus* and *E. petersi*. Ancestral character reconstruction under maximum parsimony favors lack of preference for chucks in the most recent common ancestor of *Engystomops* and the most recent common ancestor of *Engystomops* and *Physalaemus*. Similarly, the reconstruction of the proportion of females preferring the chuck results in 0.56 for the most recent common ancestor of *Engystomops* and 0.46 for the most recent common ancestor of *Engystomops* and *Physalaemus* (Fig. 4.6; binomial $P > 0.05$, at any of the experimental sample sizes). The ML reconstructions do not favor either character state at both nodes (Fig. 4.6).

Remarkably, lack of support for the sensory exploitation hypothesis persists even if the reconstruction is carried out with *E. coloradorum* coded according to Ryan & Rand's (1993b) results. Under this alternative, maximum likelihood does not support either character state in the common ancestor of *Engystomops* (proportional likelihood for preference present = 0.5; absent = 0.5); under maximum parsimony, the reconstruction favors absence. The reconstruction of the proportion of females preferring the chuck is 0.46 for the most recent common ancestor of *Engystomops* and *Physalaemus* and 0.56 for the most recent common ancestor of *Engystomops* (binomial $P > 0.05$ at any of the experimental sample sizes). Thus, neither reconstruction supports the earlier origin of the female preference compared to the male trait, as required by the sensory exploitation hypothesis. In fact, two reconstructions contradict it.

Reconstruction of the chuck evolution under parsimony indicates that it originated either in the most recent common ancestor of *E. pustulosus*, *E. freibergi*, and *E. petersi*,

or independently in each of these taxa (Fig. 4.6). Either alternative for the origin of the chuck is consistent with Ryan & Rand's (2003a) reconstruction.

The test of correlated evolution between male trait (chuck) and female preference suggests that trait and preference coevolved. Independent evolution between trait and preference was rejected with a $P < 0.01$.

Frequency allocation and matching. According to the frequency-matching hypothesis, calls with chucks are preferred because they stimulate more the BP (whine-only calls stimulate the AP only). Thus, whine-chuck (complex) calls should have a higher content of energy available for BP stimulation (above 1.5 KHz). The energy measurements do not support that prediction because complex and simple calls were not significantly different in energy content above 1.5 kHz (maximum likelihood ratio test $P = 0.390$). Simple calls of most species have more energy available for BP stimulation than *E. pustulosus* complex calls (Fig. 4.4).

The prediction of ancestral calls having less BP stimulating energy than whine-chuck calls is inconsistent with the reconstruction. The most recent common ancestor of *Engystomops* had 35.0% of its call energy above 1.5 kHz, higher than the proportion observed in a typical eastern *E. pustulosus* whine-chuck call (28.5%). A higher content is also predicted for the most recent common ancestor of *Engystomops* and *Physalaemus* (34.8%).

This result is reinforced by measurements of the strength of frequency-matching between ear tuning and call frequency. Contrary to the expectations, the closest signal-receptor match occurs in two species having whine-only calls (Table 4.3; Fig. 4.3). In

contrast, the whine-chuck call of *E. pustulosus* shows significant differences between call peak frequency and the ear's best excitatory frequency.

4.4. DISCUSSION

Sexual selection by sensory exploitation occurs when the perceptual and cognitive systems of the receiver evolve as a result of evolutionary forces operating before the origin of the sender's signal. My results are inconsistent with expectations from sensory exploitation primarily because they suggest that there is a correspondence in the origin of the female preference and the male trait (chuck).

The occurrence of sensory exploitation has been documented with varying levels of support in diverse taxonomic groups, including fish (Smith et al., 2004; Rodd et al., 2002; Basolo, 1995), arthropods (Proctor, 1992, McClintock and Uetz, 1996), and birds (Madden and Tanner, 2003; Pryke and Andersson, 2002). Sensory exploitation in túngara frogs, however, was particularly compelling because it traced phylogenetically the precedence of the bias over the trait (only three other examples are known, swordtails [Basolo, 1995], water mites [Proctor, 1992], and goodenid fish [Macías and Ramirez, 2005]). In other groups (e.g., Madden and Tanner, 2003; Rodd et al., 2002), pre-existence of the female preference before the evolution of the male trait has been implicitly accepted without historic assessments, based on assumptions of stronger selection on the sensory system in the non-sexual context compared to the mate-choice context (Rodriguez and Snedden, 2004).

My results highlight the importance of comprehensive taxon sampling to reconstruct the sequence in the evolution of preferences and traits in sexual selection. Simulations have consistently shown that the probability of correctly estimating ancestral

character states decreases as taxa are excluded from the analyses (Salisbury and Kim, 2001; Zhang and Nei, 1997). Previous accounts of sensory exploitation in túngara frogs (e.g., Ryan and Rand, 2003a) relied on a sample of female preference that excluded about 80% of the known taxa in the ingroup. As previously noted, (Gerhardt and Huber, 2002, pp. 413), confirmation of a pre-existing female preference required more comprehensive taxon sampling. Admittedly, my sample of species for female preference is still incomplete. However, it includes members of all major clades and therefore its estimates should be more reliable.

Despite sampling the same population, my results are inconsistent with Ryan & Rand's (1993b) finding of a preference for chuck-appended calls in *E. coloradorum*. The cause for this discrepancy is unknown; although it could be attributed to Ryan & Rand's choice of appending three chucks instead of one in their tests. My experiments used a single chuck to be consistent with previous tests with *E. pustulosus* (e.g., Ryan and Rand, 1990) and *P. enesefae* (Tárano & Ryan 2002) which also used a single chuck. The choice of three chucks is problematic because it hinders comparisons with experiments with one chuck. For example, while adding a single chuck to the call of *E. pustulosus* increases its energy content by 7%, adding three chucks to the call of *E. coloradorum* increases energy content by almost 100%. Although Ryan and Rand's results with *E. coloradorum* are interesting, they may reflect a qualitatively different behavioral response.

Other methodological differences are minor and include the size of the testing arena and the time before releasing the female under the funnel. Experiments also differed in the peak amplitude of the chuck relative to the whine (1.5 herein, 2.0 in Ryan & Rand).

However, differences within this range or even a larger range (0.5 vs. 2.0) do not influence female choice in *E. pustulosus* (M. J. Ryan, pers. com.)

Moreover, the lack of support for a pre-existing female preference is evident even if *E. coloradorum* is coded as showing the preference. Thus, the available evidence rejects the sensory exploitation hypothesis in túngara frogs.

There is a potential caveat with the interpretation of *P. enesefae* preference data. This species has a small area of sympatry with *E. pustulosus* where selection for species recognition could have generated reproductive character displacement resulting in a secondary loss of female preference for the heterospecific call element. However, as Ryan and Rand (2003a) pointed out, this scenario is unlikely because *E. pustulosus* chucks neither made the calls more or less attractive. Moreover, *P. enesefae* also shows a lack of preference for chucks of the allopatric *E. freibergi* (Tárano and Ryan, 2002). Using the preference data for chucks of *E. freibergi* (instead of *E. pustulosus*) does not alter the conclusions reached in my analyses (results not shown).

Correlated evolution of trait and preference. According to Ryan and Rand (1993b, pp. 265), the asynchrony in the origin of the female preference and the male trait is inconsistent with models invoking correlated co-evolution of preferences and traits like Fisherian and good-genes models of sexual selection.

However, Endler and Basolo (1998) noticed that it is possible to recover a phylogenetic correlation between trait and preference under sensory exploitation if speciation events do not occur between each preference and trait character transition. They also proposed expected patterns of character correlation on a phylogeny under alternative models of sexual selection. Specifically, Fisherian and good-genes models of sexual selection are

expected to show a correlation of trait and preference across species in a phylogeny (Kirkpatrick and Ryan, 1991; Endler and Basolo, 1998; Shaw, 1995). This correlation is evident in túngara frogs because the chuck enhances attractiveness only in the populations where it occurs. The significant phylogenetic correlation is inconsistent with sensory exploitation when the exploitative male trait evolves long after the female sensory bias originated (as proposed in túngara frogs by Ryan and Rand, 1993b). Sensory exploitation cannot be dismissed, however, when the male trait evolves rapidly after the appearance of the sensory bias in females. This scenario is suggested by bowerbirds where species-specific pre-existing sensory biases in females seem to correlate with the bower decorations of conspecific males (Madden and Tanner, 2003).

My results suggest that the evolution of the chuck can be understood under Fisherian or good-genes models of sexual selection. In a Fisherian model (Kirkpatrick, 1982), females that choose complex calls would benefit indirectly by having more attractive sons because they will produce whine-chuck calls. Under a good-genes model (Neff and Pitcher, 2005), whine-chuck calls would signal high genetic or developmental quality, and females favoring chucks will benefit indirectly by having offspring with high quality genes.

Sensory exploitation and frequency matching? The frequency-matching hypothesis predicts that complex calls (whine-chuck) are more attractive than simple calls (whine-only) because they match the inner-ear's sensory profile better (Wilczynski et al., 2001). My analyses cannot test the frequency-matching hypothesis. However, the results highlight inconsistencies with the sensory exploitation hypothesis. Contrary to expectations, my results show no correspondence between presence or absence of the

chuck and the amount of energy available for BP stimulation and the strength of the tuning with the BP. For example, *E. petersi* from Yasuní has a complex call (Boul and Ryan, 2004), but only 13% of the call energy is above 1.5 kHz and available for BP stimulation. In contrast, *E. coloradorum* has a simple call, but 38% of its call energy is above 1.5 kHz, with peak frequencies that match the best excitatory frequencies of the BP (Table 4.3; Fig. 4.3*)*. Interestingly, the simple call of *E. coloradorum* has more energy available for BP stimulation and a better tuning with the BP than *E. pustulosus* complex call.

Theory predicted that calls of *Engystomops* most recent common ancestor should have had low energy content above 1.5 KHz relative to species with whine-chuck calls. However, my results suggest that ancestral calls, as well as the calls of most contemporary species of túngara frogs, can significantly stimulate both the AP and BP. These results agree with the widespread match between auditory tuning and the frequencies emphasized in the male call among frogs (Gerhardt and Schwartz, 2001).

Túngara frog calls have been classified as complex or simple based on the chuck's presence or absence and their potential to stimulate the BP. My results show that both criteria are incongruent with each other. According to the first criterion, only the calls of *E. pustulosus*, *E. freibergi*, and those from some populations of *E. petersi* are complex; under the second criterion (BP stimulation), calls of most species might be considered complex. Future studies could benefit from abandoning this binary categorization and instead analyze continuous characters derived from acoustic measurements of the calls.

The role of central processing on mate choice decisions. Calls across species are remarkably uniform (Fig. 4.4). All species show a descending frequency sweep, which is

preceded by an amplitude-modulated component (for simplicity, I have referred to them as a single element, the whine). The frequency sweep is characterized by (i) rich harmonic structure, (ii) a dominant frequency in the first harmonic, and (iii) descending frequency and amplitude (Fig. 4.4). Except for some male *E. pustulosus*, the frequency sweep is always preceded by an amplitude-modulated prefix that has pulses varying from weakly to well defined. The prefix also has harmonic structure but its harmonics are less discrete and have a higher bandwidth. Invariably, the prefix has a higher dominant frequency than the frequency sweep (Fig. 4.2).

In calls of *E. coloradorum*, *E. pustulatus*, *E.* sp. B, and *E.* sp. D, I found a previously unknown suffix to the whine, characterized by a high dominant frequency and increased amplitude (compared to the preceding whine).

The presence of chucks, suffixes, or prefixes in túngara frog male calls improve sensory matching by maintaining energy available for BP stimulation (Fig. 4.2). Therefore, different species of túngara frogs exhibit alternative solutions to increase stimulation of the sensory system of females. An increase in BP stimulation could plausibly result in an increase female preference, but that is not necessarily the case. Although the addition of the chuck generates more intense stimulation of the peripheral auditory system, the rejection of the chuck-appended conspecific calls by female *E. randi* demonstrates that increased stimulation does not necessarily translate into increased call attractiveness and can even have the opposite effect. This finding highlights the significance of the central nervous system processing in mate choice decisions. Mate choice results from processes arranged in hierarchical stages. First, the signal is received and coded by the peripheral auditory system and then is perceived, classified, and

assessed by the central nervous system (Endler and Basolo, 1998). Experiments in *E. coloradorum* and *P. enesefae* (Tárano and Ryan, 2002) also support an active role of central processing by showing that females can be indifferent to increased peripheral stimulation (see also Wilczynski et al., 2001).

Sensory exploitation is a potentially powerful model to understand the evolutionary origin and/or maintenance of exaggerated male traits and their corresponding female preferences. Adequate hypothesis testing requires, however, comprehensive taxon sampling. The new evidence for túngara frogs shows a phylogenetic correlation between trait and preference, which is consistent with predictions from Fisherian and good genes models of sexual selection.

Table 4.1. Results for two-choice phonotaxis experiments for females. Stimuli: Wh = conspecific whine; Wh-ch = conspecific whine followed by one chuck of *E. pustulosus*. Data for *Physalaemus enesefae* from Tárano and Ryan (2002); for *E. pustulosus* from Ryan and Rand (1990). Bayesian posterior probabilities are based on priors representing five levels of preference for the wh-ch stimulus (0.90, 0.75, 0.50, 0.25, 0.10). The 0.50 prior correspond to a point of equal attractiveness between both stimuli. Bold characters signal highest probabilities.

	Call type		Proportion choosing wh-ch over wh	Bayesian posterior probabilities for wh-ch				
	Wh	Wh-ch		H: 0.90 prior	H: 0.75 prior	H: 0.50 prior	H: 0.25 prior	H: 0.10 prior
E. coloradorum	9	11	0.550	< 0.001	0.142	**0.842**	0.016	< 0.001
E. petersi Puyo	7	15	0.682	0.019	**0.759**	0.222	< 0.001	< 0.001
E. petersi La Selva	7	11	0.611	0.005	0.399	**0.591**	0.005	< 0.001
E. petersi Yasuní	2	13	0.867	**0.627**	0.366	0.008	< 0.001	< 0.001
E. pustulosus	1	19	0.950	**0.927**	0.073	< 0.001	< 0.001	< 0.001
E. randi	14	5	0.263	< 0.001	< 0.001	0.088	**0.806**	0.106
E. sp. D	13	6	0.316	< 0.001	< 0.001	0.239	**0.728**	0.032
P. enesefae	15	12	0.444	< 0.001	0.004	**0.900**	0.096	< 0.001

Table 4.2. Female choices between two alternative signals in phonotaxis experiments. The noise signal had the same amplitude envelope as the conspecific call. Values represent the number of females that chose each signal of the pair. Probabilities are from two-tailed Fisher exact tests under a null hypothesis derived for *Engystomops pustulosus* (Rand et al. 1992). A significant value indicates that females recognize the conspecific call under the experimental set up.

	Conspecific call	White noise	Fisher's exact test probability
E. coloradorum	19	2	< 0.001
E. randi	18	2	< 0.001
E. sp. D	45	1	< 0.001

Table 4.3. Sensory matching between male advertisement call and female ear frequency tuning. Best excitatory frequency (BEF-BP) represents the frequency to which the ears' basilar papilla (BP) is most sensitive (from ref. Wilczynski et al., 2001). The values of call frequency (mean ± SD) are for the high frequency peak of the male call (calls have two peaks; Fig. 4.3). Kruskal-Wallis *P*-values compare BEF-BP vs. call frequency at peak amplitude. Matching between ear tuning and call frequency is closer in *E. coloradorum*, and *E. randi* (whine-only call) than in *E. pustulosus* (whine-chuck).

	Ear BEF-BP (kHz)	Male call frequency at peak amplitude (kHz)	Difference BEF – call frequency (kHz)	Kruskal-Wallis *P*
coloradorum	2.229 ± 0.353 (*n* = 7)	2.204 ± 0.078 (*n* = 5)	-0.024	0.414
petersi (Puyo)	2.166 ± 0.046 (*n* = 4)	1.459 ± 0.167 (*n* = 7)	-0.707	0.010
pustulosus	2.133 ± 0.163 (*n* = 6)	2.545 ± 0.273 (*n* = 7)	0.412	0.037
randi	2.549 ± 0.056 (*n* = 4)	2.628 ± 0.206 (*n* = 7)	0.079	0.385
sp. B	2.259* ± 0.140 (*n* = 9)	1.890 ± 0.427 (*n* = 7)	-0.328	0.006
enesefae	2.157 ± 0.060 (*n* = 9)	0.878 ± 0.105 (*n* = 7)	-1.279	0.001

* The value originally reported by Wilczynski et al. (2001) is in error.

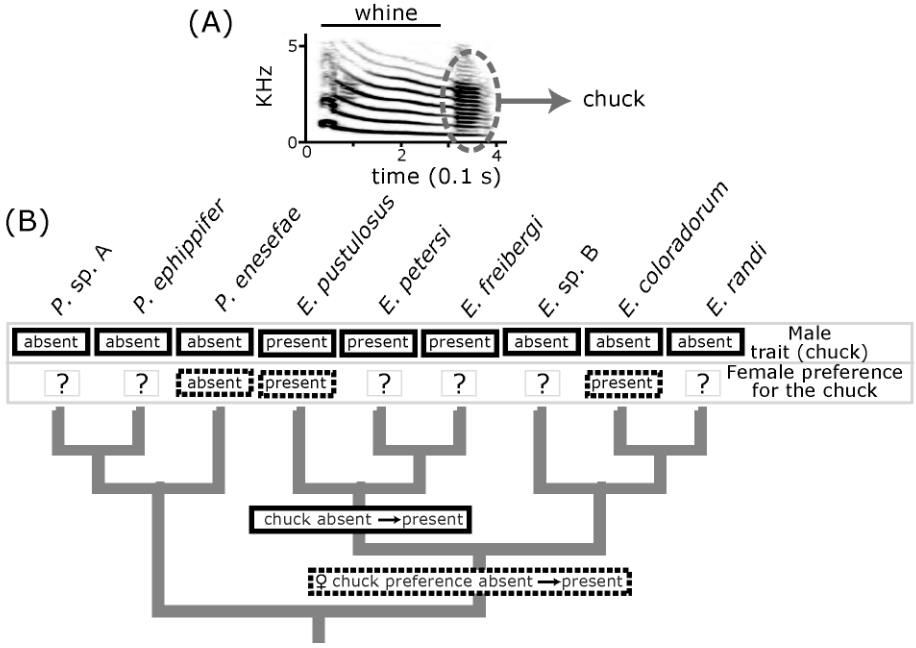


Figure 4.1. (A) Spectrogram of the advertisement call of *Engystomops pustulosus* showing the whine and the chuck. (B) Sensory exploitation hypothesis in túngara frogs as proposed by Ryan & Rand (2003a). In all species, the male call consists of a whine. In *E. pustulosus*, the call attractiveness to females can be increased by adding the chuck. Female mate choice experiments showed that the chuck of *E. pustulosus* also increases attractiveness in *E. coloradorum*. According to parsimony optimization, the preference preceded the origin of the chuck. Thus, males that produce the chuck were thought to be exploiting a preexisting sensory bias in females.

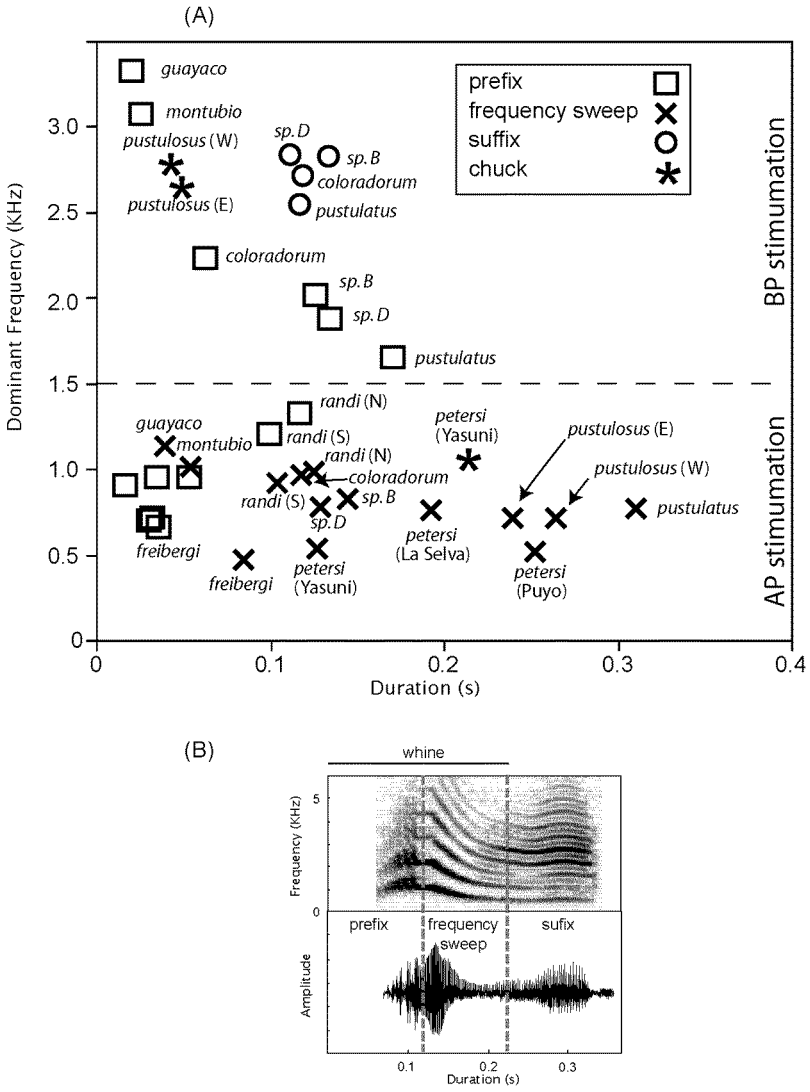


Figure 4.2. (A) Mean dominant frequency vs. mean duration of advertisement call components in *Engystomops*. Prior to the analysis, the whine was partitioned into its two acoustic components (prefix and frequency sweep). If present, the chuck or suffix are also shown. The horizontal hatched line represents an approximate threshold for sensitivity between inner-ear sensory organs (the amphibian papilla is insensitive to frequencies above 1.5 KHz, the basilar papilla is insensitive below 1.5 kHz). Note that the dominant frequency of several components are above 1.5 kHz. (B) spectrogram and oscillogram of a *E. coloradorum* call exemplifying the limits of the acoustic components (dashed vertical lines).

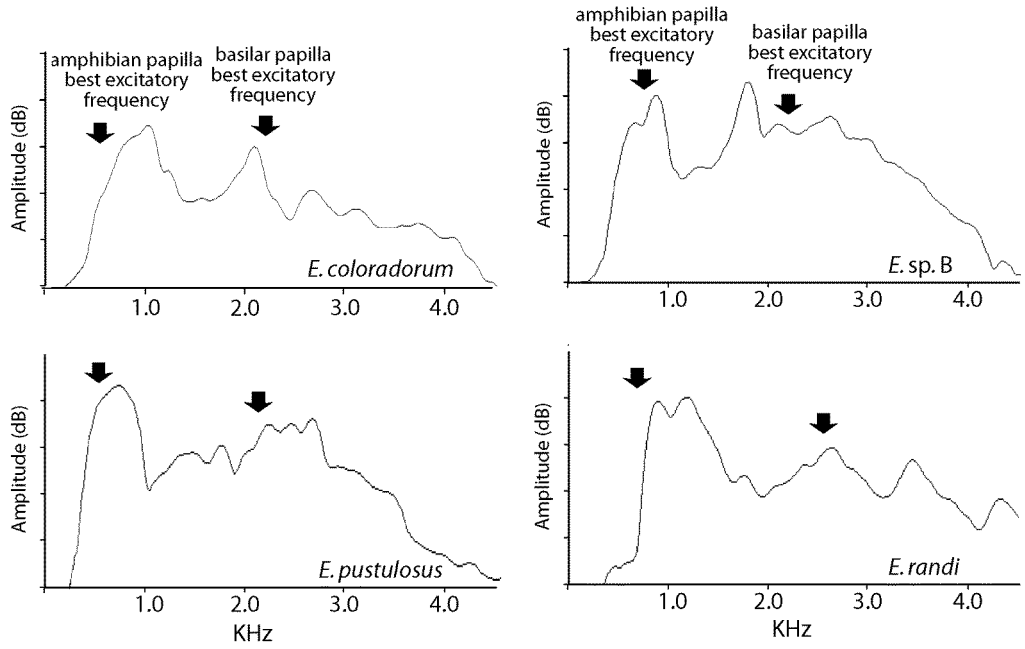


Figure 4.3. Power spectrum of typical advertisement calls of *Engystomops*. Each graph represents the distribution of sound intensity level across frequencies (from averages along the entire call). Black arrows indicate the frequencies of highest sensitivity (best excitatory frequency) of the female ear (from ref. Wilczynski et al., 2001): left arrow for the low frequency organ (amphibian papilla), right arrow for the high frequency organ (basilar papilla). *Engystomops pustulosus* call is a whine-chuck; *E. coloradorum*, *E. randi*, and *E.* sp. B. are whine-only. In *Engystomops*, the best excitatory frequencies are 0.517–0.730 KHz for the AP and 2.100–2.550 KHz for the BP (Wilczynski et al., 2001). This bimodal frequency sensitivity matches the bimodal frequency of a whine-chuck call. A similar correspondence is evident in *E. coloradorum*, *E.* sp. B, and *E. randi*.

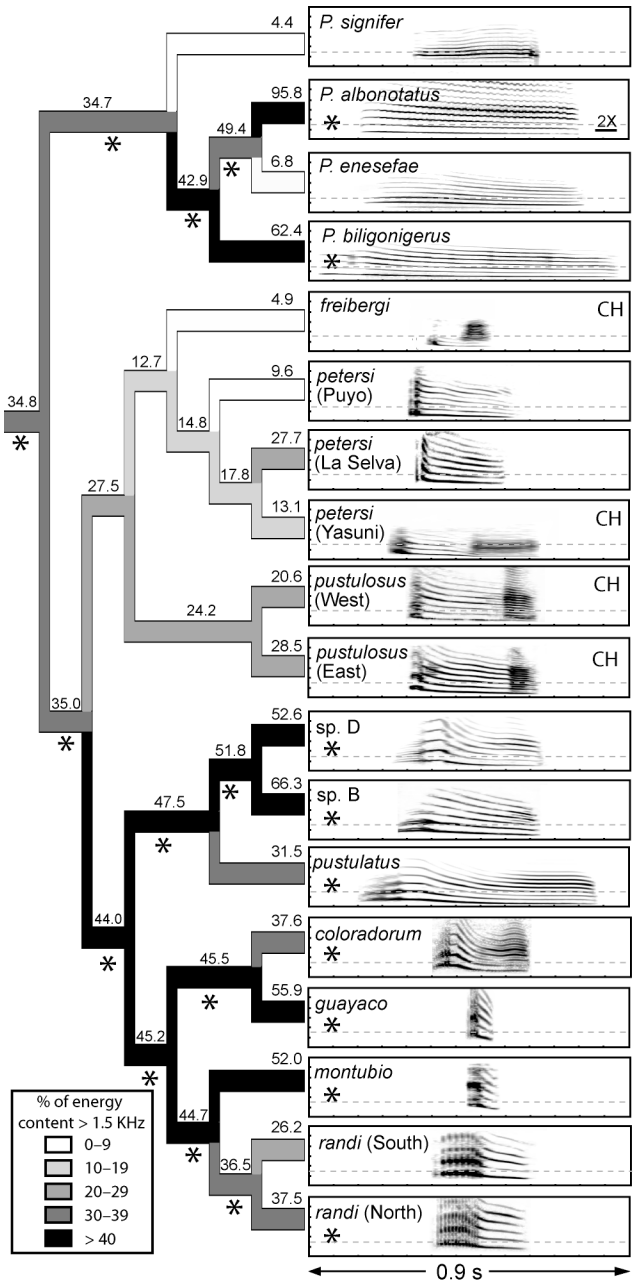


Figure 4.4. Spectrograms and energy allocation in *Physalaemus* and *Engystomops* calls. Numbers represent percentage of sound energy at > 1.5 kHz (total energy = 100%). Nodal values were reconstructed using squared-change parsimony. The percentage offers an estimate of the amount of energy available for the ear's basilar papilla (BP) stimulation (> 1.5 kHz) vs. amphibian papilla stimulation (< 1.5 kHz). Asterisks mark taxa with a content > 1.5 kHz higher than that observed on an average whine-chuck call of *E. pustulosus*; "CH" refers to taxa with whine-chuck calls. Spectrograms of calls are drawn at the same scale (box = 0.9 sec x 6 kHz) except for *P. albonotatus* (1.8 sec x 6 kHz); the horizontal dashed line shows the 1.5 kHz threshold. See text for further details.

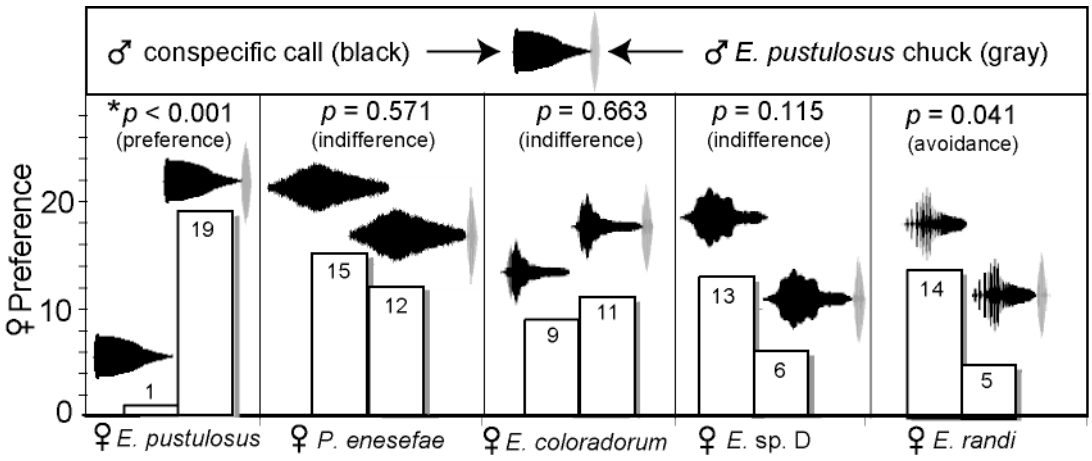


Figure 4.5. Number of phonotactic responses in túngara frogs females to two alternative male signals: their conspecific male advertisement call vs. the same call with a chuck of *Engystomops pustulosus* artificially appended. Probability values are from binomial tests (H_0 = 0.5:0.5 preference). Oscillograms of both available choices are shown above the bars. Data for *E. pustulosus* and *Physalaemus enesefae* are from Ryan & Rand (1990) and Tárano and Ryan (2002). Of these five species, only *E. pustulosus* produces chucks in nature.

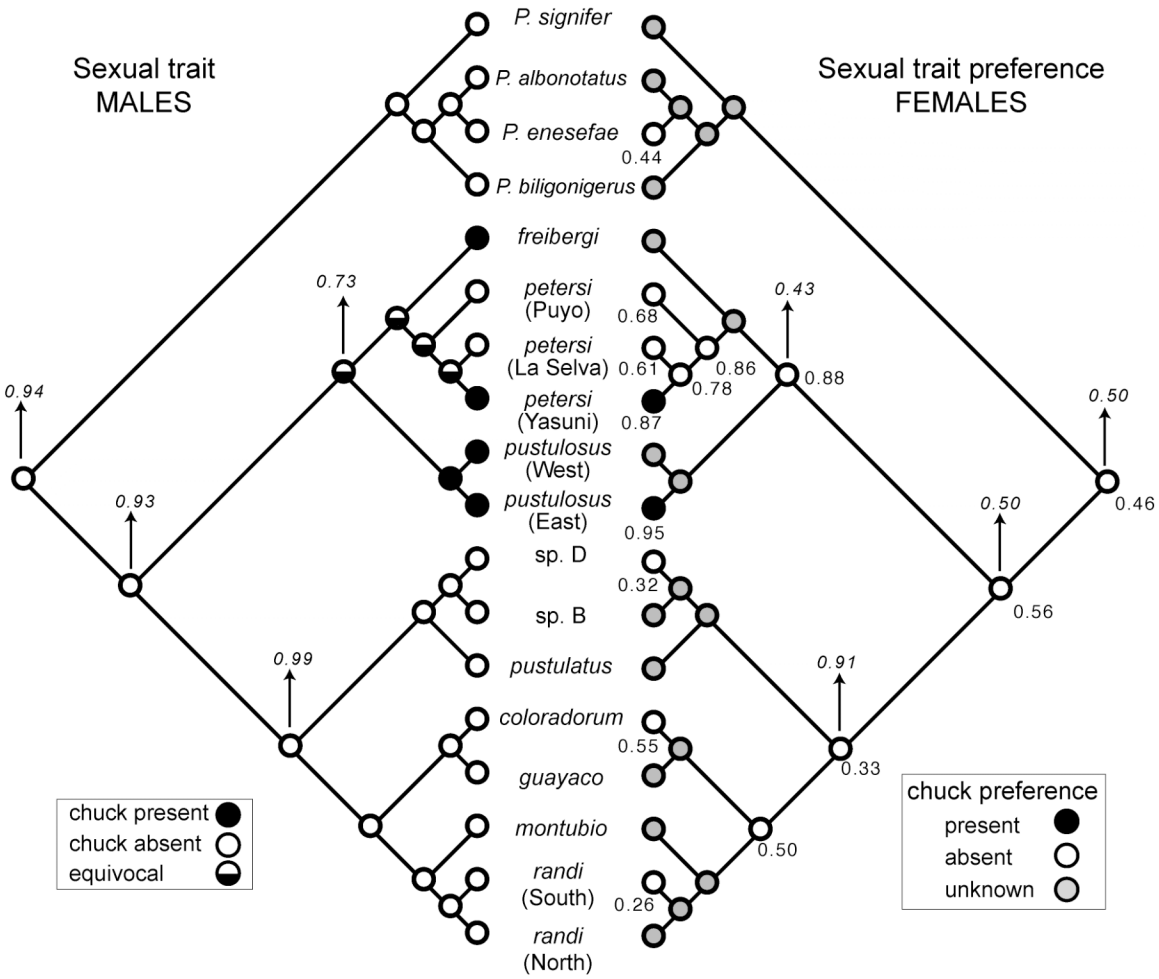
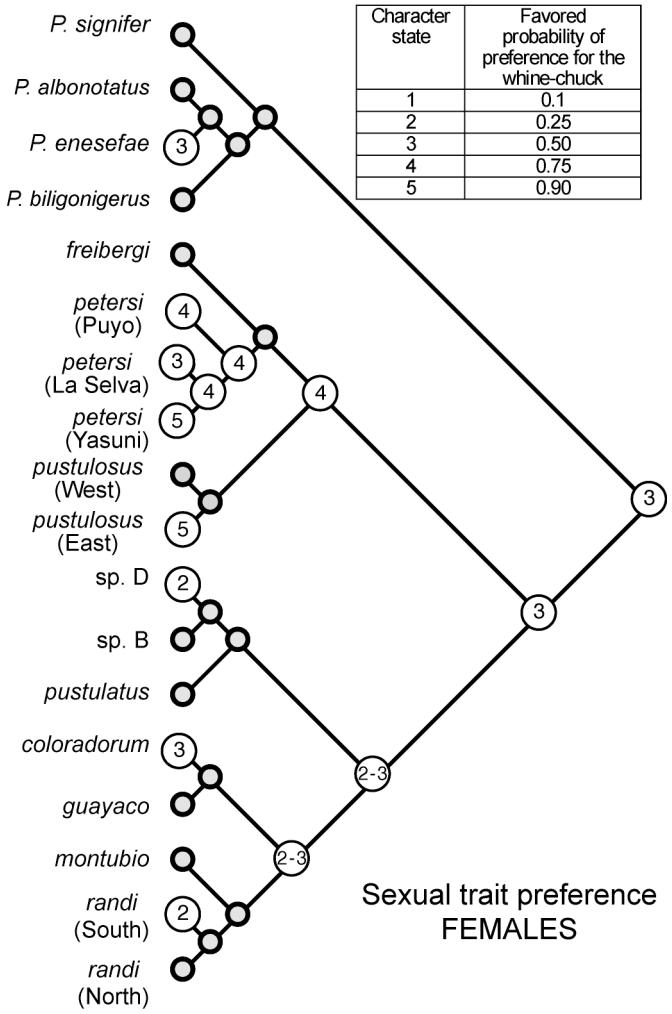


Figure 4.6. Ancestral character reconstruction using maximum parsimony and maximum likelihood for the male trait (i.e., chuck) and its corresponding female preference in túngara frogs. Circles at nodes represent the presence or absence of the character according to a parsimony reconstruction. The chuck preference by females (right tree) was obtained from experiments in which females chose between conspecific chuck-appended calls and unappended calls (Fig. 4.5). Numbers at nodes show the proportion of females preferring the chuck-appended calls in the experiments and were reconstructed with squared-change parsimony. Numbers in italics (above arrows) show the relative maximum likelihood support for the presence of chucks in the conspecific male calls (left tree) and the presence of the preference by females for chuck-appended calls (right tree). Preference data for *Engystomops pustulosus*, *E. petersi*, and *Physalaemus enesefae* are from Ryan & Rand (1990), Boul et al. (2007), Guerra (2005), and Tárano & Ryan (2002). The phylogeny is based on Ron et al. (2006).

Supplemental Data 4.1. Equivalencies of species names among publications discussing sensory exploitation in *Engystomops* (= *Physalaemus pustulosus* species group). All species in this clade were transferred from the genus *Physalaemus* to *Engystomops* by Nascimento et al. (2005). For other taxonomic changes and additions, see Ron et al. (2004; 2005; 2006). Publications are arranged in chronological order from 1990 to 2003. "E" and "S" refer to the eastern and southern range of *E. pustulosus* and *E. randi*, respectively.

	E. coloradorum	*E. freibergi*	*E. petersi*	*E. pustulatus*	*E. pustulosus* (E)	*E. randi* (S)	*E.* sp. B
Ryan & Rand 1990	*P. coloradorum*	--	--	--	*P. pustulosus*	--	--
Ryan 1990	*P. coloradorum*	*P. petersi*	?	--	*P. pustulosus*	*P. pustulatus*	--
Ryan & Rand 1993ab	*P. coloradorum*	*P. petersi*	*P. petersi*	--	*P. pustulosus*	*P. pustulatus*	*P. "pustulatus-Peru"*
Wilczynski et al. 2001	*P. coloradorum*	--	*P. petersi*	--	*P. pustulosus*	*P. pustulatus*	*P.* sp. B
Tárano and Ryan 2002	*P. coloradorum*	*P. freibergi*	*P. petersi*	*P.* sp. C	*P. pustulosus*	*P. pustulatus*	*P.* sp. B
Ryan & Rand 2003a	*P. coloradorum*	*P. freibergi*	*P. petersi*	--	*P. pustulosus*	*P. pustulatus*	*P.* sp. B

Supplemental Data 4.2. Ancestral character reconstruction of female preferences for whine-chuck calls under maximum parsimony. Character states correspond to one of five *a priori* probabilities favored by a Bayesian analysis of female choice data. The character was coded as ordered. State 3 represents no preference for whine-chuck calls; state 5 represents strong preference. Gray circles represent missing information. See text for details.



Supplemental Data 4.3. Locality data and voucher information for recordings used in call analyses. QCAZ = Museo Zoología Pontificia Universidad Católica del Ecuador, WCF = W. C. Funk field series, SC = QCAZ field series.

Taxon	Locality	Source	Voucher No.
E. coloradorum	Ecuador: Pichincha: Tinalandia	S. R. Ron	QCAZ 28668, 28682
E. freibergi	Peru: Made de Dios: Tambopata	W. C. Funk	WCF 2366–67, 5432, 2451, 2504
E. guayaco	Ecuador: Guayas: Cerro Masvale	S. R. Ron	QCAZ 19561–62, 19751, 23516, 23508
E. montubio	Ecuador: Manabí: Puerto Rico	D. C. Cannatella, S. R. Ron	QCAZ 19516–17, 19520, 19552, 19555
E. petersi (La Selva)	Ecuador: Sucumbíos: Hostería La Selva	W. C. Funk	WCF 2643, 2664–67
E. petersi (Puyo)	Ecuador: Pastaza: El Puyo	S. R. Ron	QCAZ 26249, 26252, 26254, 26257–58
E. petersi (Yasuní)	Ecuador: Orellana: Estación Científica Yasuní, Universidad Católica	K. E. Boul	--
E. pustulatus	Ecuador: Cotopaxi: La Maná	S. R. Ron	QCAZ 26519, 26651, 26709 26723–24
E. pustulosus (E)	Panama: Panama: Gamboa	X. Bernal	--

Taxon	Locality	Source	Voucher No.
E. pustulosus (W)	México: Chiapas: Tehuantepec	M. J. Ryan	--
E. randi (N)	Ecuador: Guayas: Cerro Masvale	D. C. Cannatella, S. R. Ron	QCAZ 19559–60, 19563, 19565–66
E. randi (S)	Ecuador: El Oro: Pasaje	D. C. Cannatella, S. R. Ron	QCAZ 19595, 19599, 19600, 19602–03
E. sp. B	Peru: Piura: Moropón	D. C. Cannatella, S. R. Ron	SC 16045, 16046, 16047, 16049, 16050
E. sp. D	Ecuador: El Oro: Puyango	S. R. Ron	QCAZ 26487, 26969–70, 26978, 27016
P. albonotatus	Brazil: "Caceras"	A. Cardoso	--
P. biligonigerus	Brazil: "Osario"	A. Cardoso	--
P. enesefae	Venezuela	Z. Tárano	--
P. signifer	Brazil: Río de Janeiro: Horto Forestal Santa Cruz	A. Cardoso, A. S. Rand	--

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Chapter 5

Large-scale patterns of evolution of a sexually selected signal

Abstract. Sexually selected traits are characterized by a high rate of evolution, which presumably can generate distinct macroevolutionary patterns. One possible consequence of a high rate of evolution is a rapid loss of phylogenetic signal for sexually selected traits. I tested this prediction on sexually selected traits under strong sexual selection, the advertisement calls of túngara frogs (genus *Engystomops*). I analyzed the covariance between call and phylogenetic divergence with a GLS model. In addition, I tested the hypothesis of higher divergence in calls in species with overlapping ranges relative to those completely allopatric. The results demonstrate that, contrary to the expectations, phylogenetic signal is strong in advertisement calls. A review of the literature indicates that vocalizations of birds and amphibians show significant phylogenetic signal almost without exception. Across species, *Engystomops* calls show a remarkable structural similarity with two components, a prefix and a frequency sweep, that evolve at different rates. According to two tests, call differentiation of sympatric species is not greater than that of allopatric species. I hypothesize that lack of differentiation among sympatric species results from divergence in the female preference functions rather than in male signals.

5.1 Introduction

Traits under sexual selection are among the most rapidly evolving elements of the phenotype (West-Eberhard, 1983; Mayr, 1963; Rice and Holland, 1997; Andersson, 1994). Evidence for a rapid rate of evolution of sexually selected traits encompasses diverse analytical approaches and organisms (e.g., Shaw, 1996; Ryan et al., 1990; Martins et al., 2004; Henry et al., 1999; Gleason and Ritchie, 1998). Darwin (1871) noticed, for example, that in several taxonomic groups the only diagnostic characters among closely related and otherwise cryptic species are those under sexual selection (e.g., plumage and song in birds). This pattern suggests that sexually selected traits can be among the first to diverge among populations.

If sexually selected traits change at a fast rate, it is likely that they exhibit distinct macroevolutionary characteristics. One testable prediction is that sexually selected traits exhibit less phylogenetic signal relative to other phenotypic characters. In general, if a trait changes substantially between speciation events, phylogenetic signal is lost. The strength of phylogenetic signal is inversely related to the rate of evolution because a high rate will generate homoplasy or will quickly erode synapomorphy. Phylogenetic signal also depends on time of divergence and the variety of directions in which the phenotype can evolve. If the number of possible character states of the trait is high, the probability of converging on a homoplasious state will be low.

Phylogenetic signal describes the tendency of evolutionarily related organisms to resemble each other (Blomberg and Garland, 2002). As used here, phylogenetic signal is the converse of phylogenetic lability and is similar to the concept of phylogenetic constraint (see Blomberg and Garland, 2002, for a discussion on the use of these terms).

The study of phylogenetic signal has useful practical applications. For example, it can provide insight about processes responsible for the evolution of trait diversity or it can help to decide how necessary is the application of phylogeny-based comparative methods (Freckleton et al., 2002).

Phylogenetic signal can be disrupted by selective forces that depart from a Brownian motion model of evolution. Character displacement is one of those forces because it causes closely related organisms to diverge beyond expectations (Losos, 2000). Animal communication systems that function in mate choice are vulnerable to reproductive character displacement, which occurs when the signaler, the receiver, or both diverge to avoid signal interference or maladaptive hybridization with a closely related sympatric species (e.g., Hoskin et al., 2005; Amezquita et al., 2006).

Both signal interference and reinforcement should result in more divergent communication signals among sympatric species relative to allopatric species. This prediction can be tested in groups of closely related species for which the phylogeny is known and the differentiation in the communication signal can be quantified.

Túngara frogs (genus *Engystomops*) are ideal for evaluating patterns of macroevolution of sexually selected traits and testing the expectations of signal divergence between allopatric and sympatric species pairs. In these nocturnal frogs, female mate choice is mainly based on characteristics of the advertisement call of males (Ryan, 1985; Ryan and Rand, 2003). The operational sex ratio at choruses is significantly male-biased, resulting in large variance in male reproductive success and strong sexual selection (Ryan, 1985). In addition, a well sampled and supported phylogeny for the

group is available (Ron et al., 2006) as well as recordings of the advertisement call of all known species.

Here, I analyze the macroevolutionary patterns of call variation in male túngara frogs. Using a molecular phylogeny from mitochondrial genes and the quantification of acoustic variation among all known species of *Engystomops*, I test the phylogenetic signal of their calls. I identify a common acoustic structure across species consisting of two components which I test for differences in rate of evolution. Finally, I test the hypothesis of greater signal differentiation of sympatric vs. allopatric species.

5.2 Materials and Methods

Túngara frogs (Leptodactylidae: *Engystomops*) are distributed in Central America, northern South America (east and west from the Andes) and the Amazon basin. My analysis considers all known species of *Engystomops* and six species of its sister clade (*Physalaemus* and *Eupemphix*). *Engystomops* was formerly known as the *Physalaemus pustulosus* species group.

Call recording and Analyses

Call recordings were made in the field except for one *E. coloradorum* recorded in captivity a few hours after capture. Recordings were made by K. E. Boul, D. C. Cannatella, and S. R. Ron using with a Sennheiser™ ME-67 directional microphone attached to an analog recorder SONY™ WM-DC6 or digital recorders SONY™ TCD-D8 or SONY™ MZ-NH1.

I analyzed 524 calls belonging to 143 individuals from 16 species and 20 populations. In *E. pustulosus*, *E. petersi*, and *E. randi*, I sampled more than one population because each population appears to belong to a separate species (Ron et al.,

2006). The number of individuals sampled per population ranged from 5 to 10 (see Supplemental Data 5.1 for locality data and voucher information). If available, several calls were analyzed per individual to calculate an individual average. By definition, each call is circumscribed by the sound generated by a single exhalation of air.

Calls were digitized at a sampling rate of 44 kHz and analyzed with the software CANARY 1.2.1 (Charif et al., 1995). Fast Fourier Transformation (FFT) Size was 2048. The spectral analysis had a frequency resolution of 21.5 Hz.

The acoustic characters analyzed are listed in Table 5.1. I included characters that are considered to be salient in anuran communication (Gerhardt and Huber, 2002) and several that were also evaluated by Crocroft & Ryan (1995). Among these characters, call rate, dominant frequency, and change in frequency have been demonstrated to be under sexual selection in *Engystomops* (Bosch et al., 2000; Bosch et al., 2002; Wilczynski et al., 1995).

Ambient temperature can influence the spectral and temporal features of advertisement calls (Gerhardt and Huber, 2002). My analyses of the effect of temperature on call variation did not include species in the outgroup because recording information was not available. As is often true for lowland tropical anurans, my samples show low variation in recording temperature within and among species (< 7 °C among species; < 3 °C among individuals of the same species). I did not find any significant relationship between recording temperature and the characters listed in Table 5.1 (ANOVAs from linear regressions). Thus, temperature was not considered a covariate in my analyses. Previous studies in *Engystomops* and *Physalaemus* have reported a similar lack of influence (Tarano, 2001; Ryan et al., 1996).

Test of phylogenetic signal. Analyses were based on the maximum likelihood phylogeny (including branch lengths; Fig. 3.3) presented by Ron et al. (2006). To assess phylogenetic signal in advertisement calls, I analyzed the covariance between interspecies call divergence and evolutionary relatedness among species with the generalized least squares (GLS) approach proposed by Pagel (1997). A high covariance (thus, strong signal and low lability) is expected when call divergence among species is phylogenetically conservative and compatible with a Brownian-motion model of evolution (i.e., closely related taxa have more similar calls; Pagel, 1997). In contrast, if calls have been evolutionarily labile, I expect to find significant departures from Brownian-motion. To test the phylogenetic signal of call characters, I applied the maximum likelihood model proposed by Pagel (1997) that estimates a regression coefficient, λ, between trait and total divergence. If phylogenetic signal is strong, λ will be close to 1. Alternatively, if signal is weak, λ will approach 0 (Pagel, 1999). I applied a likelihood ratio test to discriminate between a null model in which λ is forced to have a value of 0 and an alternative model in which lambda is optimized as an additional parameter. A similar test was carried out but with λ forced to 1.0 (H_0) compared to λ forced to its maximum likelihood value. The results from both tests allow discrimination among the following scenarios (Freckleton et al., 2002): (1) phylogenetic signal is absent (λ is statistically < 1 and not different from 0); (2) phylogenetic signal is strong (λ is > 0 and not different from 1), and (3) phylogenetic signal is intermediate ($0 < \lambda < 1$). Analyses were carried out with software Continuous v.1.0 (described in Pagel, 1997). I did not apply the Bonferroni sequential correction because its use is controversial and

problematic (Moran, 2003; Nakagawa, 2004). Instead, I report the probability of finding the observed number of significant tests by chance (Moran, 2003).

A subsidiary objective of the analysis of phylogenetic signal was to explore the hypothesis by Cocroft and Ryan (1995) that predicts a higher evolutionary lability in call characters under behavioral and physiological control relative to characters under morphological control. I partitioned acoustic characters into "Behavioral" and "Morphological" categories based on our current understanding of the mechanisms of call production in anurans and túngara frogs in particular (Martin, 1972; Drewry et al., 1982; Table 5.1). For example, call duration depends mainly on the contraction of muscles (abdominal and laryngeal) under central nervous system control (Martin, 1972; Schmidt, 1965). Thus, call duration and call rate are considered to be mainly under behavioral control. Alternatively, the physics of sound production dictate that frequency is a function of the mass of the vibrating structure. This generates a negative correlation between size of the vocal apparatus and calls frequency (Martin, 1972; Gerhardt and Huber, 2002). Thus, most frequency characters are considered to have a morphological basis.

Behavioral and Morphological categories, however, are not mutually exclusive because acoustic signals result from the activation of a morphological structure under behavioral control. For example, frequency modulation was categorized as behavioral because it involves the contraction of laryngeal muscles (Drewry et al., 1982). However, there is a morphological component as well because the range of the modulation is constrained by the underlying morphology. Categorization of peak time, initial and final frequency of the whine, energy content, and amplitude difference is tentative because it

partly relies on untested hypotheses of the mechanism of vocalization in *Engystomops*.

Character lability was tested on all acoustic characters and synoptic traits obtained from principal components analysis (PCA). Three PCA were carried out: (i) GLOBAL PCA including all characters listed in Table 5.1; (ii) BEHAVIOR PCA including behavioral characters only, and (iii) MORPHOLOGY PCA including morphological characters only (Table 5.1). The PCA was run in JMP 5.1 (SAS Institute, 2003). Prior to the PCA analysis, all values were squared and then log-transformed. The resulting PC scores were entered as traits in the analyses of λ.

The GLS method was also used to test for the significance of the correlation, taking phylogeny into account, between body size (measured as snout-vent length) and the call characters (Pagel, 1999). Analyses were carried out in Continuous by implementing a likelihood ratio test between two competing models. In the least parameterized null model, trait covariance is fixed to 0 while in the alternative model, it is optimized as an additional parameter. In both models, λ was estimated to its maximum likelihood value.

I tested the correlation between genetic and call distances with a randomization test. First, I calculated maximum likelihood genetic distances for all possible species pairs based on the mtDNA sequences presented by Ron et al. (2006). These values were correlated with pairwise call distances (based on GLOBAL PC I and GLOBAL PC II). The resulting correlation coefficient, *R*, was compared to a null distribution generated from 10,000 random permutations of the call distances on the genetic distances. I rejected H_o (no correlation) if the observed *R* was higher than 95% of the *R* values from the randomizations. The test was carried out with the software package Resampling

Procedures v.1.3 (Howell, 2001).

Rates of evolution. The acoustic analysis revealed that in all species of *Engystomops*, calls consist of an amplitude-modulated component (prefix) followed by a frequency sweep. I applied the rate test of phenotypic evolution (Garland, 1992) to ask whether the two elements evolved at different rates. The test estimates independent contrasts for each node in the phylogeny. Each independent contrast provides an index of the minimum amount of evolution for the descendants of each node. Contrasts from the prefix and the frequency sweep were compared with a Wilcoxon signed rank test. I analyzed four characters: call duration, frequency of the 1st spectral peak of the call, dominant frequency, and amplitude difference.

Differences in rate of evolution of frequency could result from morphological constraints. To explore this possibility, I used independent contrasts (Felsenstein, 1985) to calculate the determination coefficient (R^2) for the regression between size and each of the following parameters: (i) dominant frequency of the prefix and, (ii) dominant frequency of the frequency sweep. Analyses were carried out in Mesquite 1.12 software (Maddison and Maddison, 2005a) with the PDAP module (version 1.08; Midford et al., 2003).

Call divergence: sympatry vs. allopatry. Species assortment or selection due to signal interference or reinforcement should result in increased divergence in the calls of sympatric species pairs relative to allopatric species pairs (Duellman and Pyles, 1983; Gerhardt, 1994). To test this prediction, all possible species pairs were categorized as sympatric or allopatric (sympatric if any portion of their distribution ranges overlapped). I calculated call Euclidean distances between species pairs based on the scores of PCs

with Eigenvalues > 1 from the GLOBAL PCA.

Based on those distances, two types of tests were carried out. The first type was a randomization test in which a Student's *t*-statistic was calculated for the differences between allopatric and sympatric pairs. This *t*-value was compared to a null distribution of *t* generated from 10,000 random assignments of the call distances to the allopatric and sympatric categories. I rejected H_o (call differences between sympatric pairs are not different from allopatric pairs) if the observed *t* value was higher than 95% of the *t* values from the randomizations. The test was carried out with Resampling Procedures software v.1.3 (Howell, 2001). Each principal component was tested individually.

The randomization test described above does not take evolutionary history into account. I developed a second test, which incorporates a phylogenetic component in the comparison of sympatric and allopatric divergence. First, I calculated Mann-Whitney's *Z* statistic for the call distances of allopatric vs. sympatric species pairs (based on PC scores from PCA GLOBAL). This *Z* value was compared with a null distribution of *Z* generated from 500 simulations of a character evolving under a Brownian-motion model of evolution along the phylogeny (including branch lengths) presented by Ron et al. (2006). Under this model, the expected change of a trait is distributed normally with a variance proportional to branch length times a fixed rate. Thus, the trait distance between any pair of species in the phylogeny is inversely proportional to the time since they shared a common ancestor. Under the null model, trait distances between sympatric pairs should not be higher than those expected from their divergence in the phylogeny. For each simulation, I estimated *Z* values with the same procedure used for the real characters. I rejected H_o (call differences between the sympatric and allopatric categories is not higher

than expected under Brownian-motion) if the observed *Z* value was higher than 95% of the *Z* values from the simulations. Each PC was tested individually. Character simulations were implemented in Mesquite v.1.12 (Maddison and Maddison, 2005a) with the StochChar module (Maddison and Maddison, 2005b). For both tests, calls were recorded in localities of sympatry except for the pairs *P. biligonigerus*-*P. albonotatus*, *P. biligonigerus*-*E. nattereri*, and *P. enesefae*-*E. pustulosus*.

5.3 Results

Phylogenetic signal in advertisement calls. My analysis shows that calls have significant phylogenetic signal. According to the maximum likelihood ratio tests, λ is significantly different from 0 in 18 out of 19 characters (including all PCs; Table 5.2). In general, calls of closely related species tend to be similar to each other (Fig. 5.1). All characters show a significant influence from phylogeny but some of them depart from a perfect fit. In total, λ values of 8 characters were significantly different from 1. Most of those characters are morphology-based, including GLOBAL PC I, which loads mainly in morphology-based characters, and MORPHOLOGY PC I (Table 5.2). In contrast, GLOBAL PC II, a component that loads mainly in behavioral characters (Table 5.3) has a tight correlation with phylogeny (λ not different from 1). BEHAVIOR PC I and most characters based on behavior also show a strong correlation. The observed trends are unlikely to be an artifact of multiple tests because the chance probability of obtaining 18 significant results out of 19 tests at $\alpha = 0.05$ is 6.88×10^{-23}; the probability for 8 out of 19 tests is 1.69×10^{-6}.

Body size is strongly correlated with all characters that measure call frequency except energy content and dominant frequency (Table 5.4). All other characters were

uncorrelated. Size has a strong phylogenetic signal. Its λ is significantly different from 0 and not significantly different from 1 ($P = 0.002$ and 0.1381, respectively; $\lambda = 0.81$).

There is a significant relationship between call and genetic distance according to the randomization tests (for GLOBAL PC I, $P < 0.001$; for GLOBAL PC II, $P < 0.001$). Inspection of Figure 5.2 shows non-random covariation between call and genetic distance: species pairs with low genetic distances do not have highly divergent calls; pairs with high genetic distances vary between low and high divergence.

Evolution of acoustic structure. Calls of *Engystomops* share a similar acoustic structure (Fig. 5.3). In all cases, there is a downward frequency sweep characterized by (i) rich harmonic structure, (ii) dominant frequency in the first harmonic, and (iii) descending frequency and amplitude (Fig. 5.3). The *Engystomops* clade is also characterized by the presence of a prefix before the frequency sweep.

The prefix has pulses varying from weakly to well-defined and its harmonics are less discrete and have a higher bandwidth that those of the frequency sweep. The duration of the prefix varies between 6% and 48% of the duration of the entire call (in *E. pustulosus* and *E. randi*, respectively; Table 5.5). Invariably, the prefix has a higher dominant frequency than the frequency sweep (Fig. 4.2A). The prefix is only absent in some male *E. pustulosus*.

The calls of *Physalaemus* and *Eupemphix* also have the downward frequency sweep, except in *P. signifer* (slight upward sweep). Unlike *Engystomops*, the first harmonic in *Physalaemus* and *Eupemphix* does not carry the most energy. Most energy is in the third harmonic in *P. signifer* and the second in *Eupemphix nattereri* and *P. enesefae*. In *P. albonotatus*, *P. barrioi* and *P. biligonigerus*, the dominant frequency

changes from the lower (1–2) harmonics at the beginning of the call to the upper harmonics (4–7) at the end. In all *Physalaemus* and *Eupemphix* calls, the prefix is absent. There is significant call differentiation between *Engystomops* and its sister clade (Fig. 5.4). Values for representative acoustic characters across all species are shown in Table 5.5.

Rates of evolution. There is a higher variability in duration of the frequency sweep relative to the prefix (Fig. 4.2A). The prefix, in contrast, is more variable in dominant frequency. The rate tests of phenotypic evolution suggest that the prefix and the frequency sweep evolved at different rates. The prefix had a higher rate of evolution in fundamental frequency (Z = -28.5, P = 0.024), and dominant frequency (Z = -40.5, P = 0.001); the frequency sweep had a higher rate in duration (Z = 28.5, P = 0.024). The rate was not different for amplitude differential (Z = -0.5, P = 0.500).

Size has greater explanatory power of the variation of the frequency sweep than the prefix (R^2 = 0.53 and 0.26, respectively, for linear regression; R^2 = 0.16 and 0.04, for independent contrasts).

Call divergence sympatry vs. allopatry. According to both tests, call differentiation of sympatric species is not greater than that of allopatric species (Table 5.6). The phylogeny-based tests comparing call distances of sympatric species pairs vs. allopatric species pairs show lack of significant differences (Table 5.6). Lack of differentiation is also evident in the relationship between call and genetic distances (Fig. 5.2). The randomization tests provided a similar result: call differences between sympatric species pairs are not statistically different from those of allopatric species.

5.4 Discussion

Phylogenetic signal. My analyses indicate that the evolution of advertisement calls has been phylogenetically conservative. Influence of phylogeny in call structure is confirmed by high values of λ, which in all but one character were significantly different from 0 (Table 5.2). The most divergent clades in the analysis (*Engystomops* vs. *Physalaemus*) segregate almost completely in call space (Fig. 5.4). This pattern is expected for characters that are phylogenetically informative and have low homoplasy.

It is unlikely that the predominance of phylogenetic signal among the characters analyzed is an artifact of the GLS model. Studies based on this model report a significant proportion of characters lacking phylogenetic signal. For example, Freckleton et al. (2002), estimated λ values for 106 ecological characters for a diverse array of taxa and found that 40% of them were not significantly different from 0. Simulations have shown that these tests typically have low rates of Type I error (Freckleton et al., 2002).

The strong correlation of phylogeny with signal structure in *Engystomops* is inconsistent with a previous analysis that reported large incongruences between a phylogeny based on call characters and another based on molecular markers, morphology, and allozymes (Cannatella et al. 1998). The disagreement could result from differences in the quantification of call variables or taxon sampling. Further, my analysis considers all harmonics in the advertisement call, whereas Cannatella et al. (1998) analyzed only the first harmonic of the frequency sweep. In addition, my analysis has a more complete taxon sampling and includes highly divergent advertisement calls that were previously unavailable (e.g., *E. guayaco*, *E. randi*).

Despite the significant phylogenetic signal, evidence suggests that the advertisement call of túngara frogs evolved at fast rates relative to other phenotypic traits. For example, closely related species (e.g., *E. guayaco*, *E. randi*, *E. montubio*) tend to be morphologically cryptic and can not be reliably recognized without genetic or call data (Ron et al., 2004; Ron et al., 2005). In addition, numerous studies have shown that calls in *Engystomops* and *Physalaemus* are under sexual selection (e.g., Bosch et al., 2002; Rand and Ryan, 1981) and sexually selected traits tend to evolve at a high rate (West-Eberhard, 1983; Andersson, 1994). Finding significant phylogenetic signal despite a presumably high rate of evolution could be a result of large possibilities for change in acoustic space or pleiotropic effects from phylogenetically conservative traits like body size (see below).

How representative is the pattern of phylogenetic signal recovered in túngara frog calls relative to other taxa and trait types? Few studies have analyzed phylogenetic signal of sexually selected traits in a rigorous statistical framework (e.g., Martins et al., 2004). Most reports of phylogenetic signal are based on assessments of the fit of sexually selected traits to phylogenies based on independent data sets (e.g., Price and Lanyon, 2002; Kusmierksi et al., 1997). Comparisons among studies are difficult because of differences in analytical approaches, statistical rigor, density of taxon sampling, and time scale. However, a review of the a literature can elucidate general trends on the distribution of phylogenetic signal. Supplemental Data 5.2 presents a list of those studies. They have been categorized as showing strong, intermediate, or weak phylogenetic signal, based on the conclusions of each study's authors. In some cases, the studies do not

discuss phylogenetic signal explicitly but instead refer to phylogenetic informativeness or levels of homoplasy in traits.

Out of 19 studies listed, 8 report strong phylogenetic signal, 2 intermediate, and 9 weak. A majority of the studies of traits other than vocalizations fit the expectations of rapid change and weak phylogenetic signal under sexual selection (Supplemental Data 5.2). For example, variation of head bob displays in lizards is characterized by low phylogenetic information and large inter-specific differences, even among closely related taxa (Martins and Lamont, 1998; Martins et al., 2004; Ord and Martins, 2006). Similar patterns characterize the evolution of bower design in bowerbirds (Kusmierksi et al., 1997), and courtship songs in *Drosophila* (Gleason and Ritchie, 1998) and lacewings (Henry et al., 1999).

Strong phylogenetic signal was more frequent among studies of vocalizations: 6 out of 8 studies report strong signal. For example, vocalizations of oropéndolas and kinglets (*Regulus*) are characterized by low levels of homoplasy and high congruence with phylogeny (Päckert et al., 2003; Price and Lanyon, 2002). Similarly, in a review of four bird clades, Irwin (1996) reported general agreement between phylogenies based on vocal and visual displays with those based on morphological characters.

In amphibians, the only analysis available (Cocroft and Ryan 1995) reports that several acoustic traits in the calls of *Bufo* and *Pseudacris* are phylogenetically conservative and are consistent with my results. The combined evidence demonstrates that vocalizations of frogs and birds are significantly influenced by phylogenetic history.

I hypothesize that the strong phylogenetic signal that characterizes the vocalizations of amphibians and birds can be partly attributable to size constraints. Spectral properties

of calls are significantly and negatively correlated with size in my dataset. This relationship is consistent with the well documented correlation between size and the spectral properties of vocalizations in birds and amphibians (Ryan and Brenowitz, 1985; Gerhardt and Huber, 2002).

A correlation between body size and phylogeny has been demonstrated in a wide variety of organisms, almost without exception (e.g., Blomberg et al., 2003; Freckleton et al., 2002; Phillimore et al., 2006). Because size has a very strong phylogenetic signal, it is likely that the phylogenetic signal detected in vocalizations of birds and amphibians is a byproduct of their correlation with size. Evidently, alternative processes should be responsible for the signal recovered in characters that are uncorrelated with size like call duration (Table 5.4).

Ryan (1988) distinguished between two broad patterns of covariation between phylogeny and traits of advertisement calls. He proposed that traits that are under active control and are heavily influenced by behavior and physiology are highly labile and show low congruence with phylogeny. These traits are usually temporal (e.g., call duration). In contrast, traits that broadly depend on the morphology of the vocal apparatus, usually spectral or frequency-related, are more conservative and show higher covariation with phylogeny. This hypothesis was tested by Cocroft & Ryan (1995) on frogs of the genera *Bufo* and *Pseudacris*. Their analyses showed support for the hypothesis in *Pseudacris* but did not find a significant difference in *Bufo*.

According to Blomberg et al. (2003), behavioral characters are more labile than morphological characters. This generalization is consistent with Cocroft and Ryan's (1995) hypothesis. In *Engystomops*, however, I found more lability in morphological

characters. Although unexpected, this result is the only unambiguous test of Cocroft and Ryan's hypothesis in a clade other than *Pseudacris*. My result is also consistent with a recent analysis in birds (Icteridae) showing that spectral characters of the songs are more variable and less phylogenetically informative than temporal characters (Price and Lanyon, 2002). Differences in phylogenetic signal between both character types needs to be tested in additional groups.

The evolution of acoustic structure. Call variation in *Engystomops* and *Physalaemus* revolves around a common structural design. With only one exception, all species analyzed share a downward frequency sweep. In *Engystomops*, the frequency sweep is preceded by an amplitude-modulated prefix. This structural template has been maintained for the last 15 million years, according to a genetic estimate (Weigt et al., 2005).

In *Engystomops*, the dominant harmonic in the frequency sweep is always the first and has low interspecific variability in dominant frequency and high variability in duration. (Fig. 4.2A). The prefix shows the opposite pattern: it varies widely in dominant frequency and it is more conservative in duration (Fig. 4.2A). These patterns are consistent with the analyses of rates of evolution. While the prefix has evolved faster in frequency, the frequency sweep has evolved faster in duration. These results demonstrate that the advertisement call has not evolved in a unitary fashion. Instead, the two distinctive components have been changing at different rates along separate acoustic axes. Two non-exclusive hypotheses can explain the differences in evolutionary rate: (1) differential selective regimes, or (2) differential morphological or developmental constraints. In the following section, I discus these two hypotheses.

Females exert a strong selective force on the advertisement call of frogs (Gerhardt and Huber, 2002). Experiments in *E. pustulosus* have demonstrated that females discriminate and recognize calls based on duration, inter-call interval, dominant frequency, sound amplitude, and other traits (Wilczynski et al., 1995; Ryan, 1985; Bosch et al., 2000). The differences in rate of evolution in frequency between the frequency sweep and the prefix could be explained by the female's recognition and discrimination rules. In *E. pustulosus* females do not recognize the call unless the frequency sweep is present (Ryan, 1985). The frequency sweep stimulates primarily one of two auditory organs in the inner ear, the amphibian papilla (AP). Thus, AP stimulation seems necessary to elicit a female response. Measurements of best excitatory frequencies of the AP in *Engystomops* are constrained to a narrow range, between 516 and 730 Hz and AP sensitivity is restricted to frequencies below 1100 Hz (Wilczynski et al., 2001). Thus, the critical role of the frequency sweep for call recognition could constrain it to evolve within a narrow parameter space circumscribed by the frequency sensitivity of the AP (Fig. 4.2A).

Under the second hypothesis, differences in rates of evolution could be attributable to morphological constraints. If so, one would predict a stronger correlation between morphology and frequency in the whine relative to the prefix. This hypothesis is supported by the data: size is more strongly correlated with the dominant frequency of the frequency sweep than the dominant frequency of the prefix. Thus, it is likely that the prefix has evolved faster in frequency because it is less dependent on the underlying morphological structure.

Call divergence sympatry vs. allopatry. Contrary to the expectations, differentiation

between calls of sympatric species is similar to that of allopatric species (Table 5.6). Thus, there is no evidence of reproductive character displacement or signal-based species assortment. A higher differentiation in sympatry was expected as a result of the potential fitness cost of females mating with heterospecific males or signal interference (Duellman and Pyles, 1983; Gerhardt, 1994).

Fitness costs due to hybridization can be avoided by divergence in the sender's signals, divergence in the receiver's preferences, or both (e.g., Hoskin et al., 2005; Höbel and Gerhardt, 2003). For example, reproductive character displacement of female preferences, instead of male calls, was reported in *Hyla cinerea* (Höbel and Gerhardt, 2003). This possibility is suggested by the fact that calls of sympatric species of *Engystomops* are markedly different. Call differentiation beyond expectations from Brownian motion may be unnecessary beyond a threshold above which the evolution of more discriminant preferences has low cost.

Table 5.1. Call traits analyzed in this study. "Type" indicates whether the trait is behavioral (B) or morphological (M). *Engystomops pustulosus* and some populations of the *E. petersi* species complex are able to add a facultative high-frequency suffix to their call ("chuck" or "squawk"). A comparable suffix is also produced by *E. coloradorum*, *E. pustulatus*, *E.* sp. B, and *E.* sp. D. Measurements that involve the complete call include the suffixes.

Character	Type	Description
Call duration	B	Time from the beginning to the end of the call
Call rate	B	No. of calls/second
Whine duration	B	From the beginning to the end of the whine
Peak time	B	Time from the beginning of the call to the point of its maximum amplitude (rise time)
Call shape	B	Peak time/call duration
Frequency modulation	B	(change in frequency 1st harmonic)/call duration
Change in frequency 1st harmonic	B	final frequency – initial frequency of the whine
Initial frequency 1st harmonic	B	Initial frequency of the whine
Call energy content	M	Proportion energy above 1.5 kHz/energy below 1.5 kHz
Frequency of the 1st spectral peak of the call	M	Frequency of the 1st spectral peak in power spectrum of the entire call (fundamental frequency)
Frequency of the 2nd spectral peak of the call	M	Frequency of the 2nd spectral peak in power spectrum of the entire call
Frequency of the 1st spectral peak of the whine	M	Frequency of the 1st spectral peak in power spectrum of the entire whine (fundamental frequency)

Character	Type	Description
Amplitude difference	M	Difference in amplitude (dB) between the 1st and 2nd spectral peaks in a power spectrum of the entire call
Final frequency	M	Frequency at the end of the first harmonic

Table 5.2. Correlations of acoustic characters with phylogeny (λ). The parameter λ was calculated with a GLS model and generally varies between 0 (character has evolved independently of phylogeny) and 1 (perfect fit with phylogeny). Characters under behavioral and morphological control are referred with B and M, respectively. Bold numbers indicate $P < 0.05$.

Character	λ	Probability $H_{0:}$ λ = 1	Probability $H_{0:}$ λ = 0
GLOBAL PC I (Mainly morphology)	0.882	**0.021**	**< 0.001**
GLOBAL PC II (Mainly behavior)	0.920	0.088	**0.003**
GLOBAL PC III (Mainly behavior)	0.850	**0.021**	**0.011**
BEHAVIOR PC I	0.909	0.411	**0.001**
MORPHOLOGY PC I	0.879	**0.013**	**< 0.001**
Call duration (B)	0.923	0.833	**0.045**
Call rate (B)	0.877	0.110	**0.018**
Whine duration (B)	0.903	0.196	**0.001**
Peak time (B)	0.998	0.972	**0.003**
Call shape (B)	0.922	0.182	**0.015**
Frequency modulation (B)	0.945	0.392	**< 0.001**
Change in frequency 1st harmonic (B)	0.991	0.759	**< 0.001**
Initial frequency 1st harmonic (B)	0.832	**0.002**	**< 0.001**
Call energy content (M)	0.936	0.183	**0.006**
Frequency of the 1st spectral peak of the call (M)	0.918	**0.036**	**< 0.001**
Frequency of the 2nd spectral peak of the call (M)	0.887	**0.032**	**0.005**
Frequency of the 1st spectral peak of the whine (M)	0.878	**0.009**	**< 0.001**
Amplitude difference (M)	0.934	0.267	0.068
Final frequency (M)	0.625	**< 0.001**	**0.012**

Table 5.3. Character loadings for principal components analysis applied to acoustic characters of calls of *Engystomops* and six outgroup species. Three separate analysis were carried out. The GLOBAL analysis combines all acoustic characters. The BEHAVIOR analysis includes characters that are mostly under behavioral control. The MORPHOLOGY analysis includes characters that are heavily influenced by the morphological structure of the vocal apparatus. Bold figures show characters with high loadings.

Character	PC I	PC II	PC III
GLOBAL PCA			
Call duration (B)	-0.133	**0.490**	-0.071
Call rate (B)	0.122	**-0.380**	**0.384**
Whine duration (B)	-0.195	**0.431**	-0.036
Peak time (B)	-0.159	0.364	**0.412**
Call shape (B)	-0.091	0.014	**0.672**
Frequency modulation (B)	0.311	-0.240	-0.155
Change in frequency 1st harmonic (B)	0.284	0.214	-0.267
Initial frequency 1st harmonic (B)	**0.360**	0.124	0.032
Call energy content (M)	0.264	0.343	0.102
Frequency of the 1st spectral peak of the call (M)	**0.364**	0.059	0.033
Frequency of the 2nd spectral peak of the call (M)	0.310	0.191	0.136
Frequency of the 1st spectral peak of the whine (M)	**0.364**	0.090	0.080
Amplitude difference (M)	-0.249	-0.108	0.092
Final frequency (M)	0.309	-0.022	0.288
Eigenvalue	7.053	3.485	1.857
Percentage of explained variance	50.382	24.890	13.266
BEHAVIOR PCA			
Call duration (B)	**0.421**	0.328	0.085

Character	PC I	PC II	PC III
Call rate (B)	-0.314	**-0.426**	0.256
Whine duration (B)	**0.452**	0.210	-0.037
Peak time (B)	0.414	-0.054	**0.477**
Call shape (B)	0.159	**-0.421**	**0.601**
Frequency modulation (B)	**-0.467**	0.154	0.098
Change in frequency 1st harmonic (B)	-0.181	**0.561**	0.293
Initial frequency 1st harmonic (B)	-0.270	0.384	**0.492**
Eigenvalue	4.070	2.269	1.280
Percentage of explained variance	50.87	28.36	16.00
MORPHOLOGY PCA			
Call energy content (M)	0.402	**-0.262**	**0.669**
Frequency of the 1st spectral peak of the call (M)	**0.443**	0.074	-0.236
Frequency of the 2nd spectral peak of the call (M)	**0.430**	0.136	**0.442**
Frequency of the 1st spectral peak of the whine (M)	**0.457**	0.104	**-0.365**
Amplitude difference (M)	-0.305	**0.787**	0.355
Final frequency (M)	0.393	**0.526**	-0.207
Eigenvalue	4.569	0.812	0.386
Percentage of explained variance	76.14	13.53	6.44

Table 5.4. Correlations between body size and call characters. Analyses account for phylogenetic history and are based on Pagel's (1999) GLS method. The chance probability of obtaining 5 significant results out of 15 tests at $\alpha = 0.05$ is 0.0006.

Character	Regression coefficient	*P*
Call duration (B)	< 0.001	0.997
Call rate (B)	0.204	0.416
Whine duration (B)	-0.205	0.401
Peak time (B)	-0.021	0.929
Call shape (B)	-0.043	0.856
Frequency modulation (B)	-0.176	0.457
Change in frequency 1st harmonic (B)	0.037	0.879
Initial frequency 1st harmonic (B)	-0.540	**0.016**
Call energy content (M)	-0.265	0.270
Frequency of the 1st spectral peak of the call (M)	-0.556	**0.011**
Frequency of the 2nd spectral peak of the call (M)	-0.613	**0.004**
Frequency of the 1st spectral peak of the whine (M)	-0.479	**0.037**
Amplitude difference (M)	-0.026	0.922
Final frequency (M)	-0.648	**0.002**
Dominant frequency of the call	-0.349	0.125

Table 5.5. Summary advertisement call measurements (mean ± SE) for *Engystomops*. See Table 5.1 for definitions of characters. Frequency units are Hz.

	N	Call duration (sec)	Prefix duration (sec)	Call rate (sec^{-1})	Peak time (sec)	Frequency modulation	Energy content	Frequency 1^{st} peak	Dominant frequency	Final frequency
E. coloradorum	5	0.299 ±0.013	0.063 ±0.008	0.539 ±0.109	0.059 ±0.004	–492.6 ±23.18	0.377 ±0.054	1023.7 ±24.7	1023.7 ±24.7	497.95 ±15.31
E. freibergi	7	0.113 ±0.008	0.034 ±0.007	0.334 ±0.09	0.025 ±0.002	–165.3 ±10.4	0.049 ±0.011	577.5 ±21.2	577.6 ±21.2	363.35 ±8.31
E. guayaco	7	0.0610 ±0.002	0.021 ±0.002	4.055 ±0.205	0.0140 ±0.001	–373.9 ±39.4	0.559 ±0.059	1142.0 ±48.1	3283.8 ±52.4	803.36 ±9.02
E. montubio	7	0.080 ±0.002	0.025 ±0.004	3.759 ±0.095	0.0186 ±0.001	–311.1 ±12.3	0.52 ±0.077	994.8 ±22.6	994.8 ±22.6	726.54 ±15.37
E. petersi La Selva	7	0.258 ±0.014	0.051 ±0.011	0.41 ±0.043	0.0305 ±0.003	–345.8 ±18.9	0.277 ±0.041	829.5 ±18.7	829.5 ±18.7	592.16 ±15.04
E. petersi Puyo	7	0.289 ±0.011	0.033 ±0.005	0.187 ±0.026	0.022 ±0.001	–189.2 ±9.7	0.096 ±0.027	621.0 ±44.6	621 ±44.6	374.23 ±7.90
E. petersi Yasuni	7	0.355 ±0.015	0.028 ±0.005	0.612 ±0.103	0.020 ±0.001	–287.5 ±11.6	0.131 ±0.049	598.25 ±20.92	598.3 ±20.9	277.21 ±9.92
E. pustulatus	10	0.592	0.171	0.428	0.165	–405.7	0.315	816.5	816.5	428.04

	N	Call duration (sec)	Prefix duration (sec)	Call rate (sec^{-1})	Peak time (sec)	Frequency modulation	Energy content	Frequency 1st peak	Dominant frequency	Final frequency
		±0.016	±0.024	±0.079	±0.007	±11.0	±0.065	±14.72	±14.7	±12.38
E. pustulosus E	7	0.371	0.038	0.433	0.0341	–376.0	0.285	720.4	720.4	459.42
		±0.016	±0.022	±0.035	±0.007	±8.6	±0.051	±18.6	±18.6	±12.60
E. pustulosus W	7	0.293	0.017	--	0.015	–402.6	0.206	742.7	742.7	481.32
		±0.017	±0.011		±0.006	±27.8	±0.025	±44.4	±44.4	±7.15
E. randi N	7	0.239	0.114	1.722	0.067	–247.4	0.375	1162.9	1162.9	819.95
		±0.005	±0.011	±0.049	±0.00295	±10.9	±0.071	±53.8	±53.8	±11.97
E. randi S	7	0.206	0.098	1.889	0.065	–236.7	0.263	1041.2	1041.2	777.60
		±0.005	±0.014	±0.127	±0.00429	±24.5	±0.062	±41.6	±41.6	±11.16
E. sp. B	7	0.405	0.131	0.624	0.128	–451.4	0.663	872.2	1889.6	458.55
		±0.017	±0.01	±0.046	±0.004	±11.1	±0.046	±17.0	±63	±12.58
E. sp. D	10	0.367	0.126	0.676	0.119	–449.0	0.526	858.88	2018.7	467.58
		±0.016	±0.015	±0.023	±0.005	±14.4	±0.03	±33.51	±12.1	±8.13
Eu. nattereri	7	0.088	--	3.926	0.033	–38.9	0.022	331.47	711.8	310.85
		±0.005		±0.124	±0.003	±8.6	±0.006	±8.45	±44.1	±7.426

	N	Call duration (sec)	Prefix duration (sec)	Call rate (sec^{-1})	Peak time (sec)	Frequency modulation	Energy content	Frequency 1st peak	Dominant frequency	Final frequency
P. barrioi	7	1.041 ±0.044	--	0.094 ±0.018	0.046 ±0.003	–298.2 ±17.8	0.526 ±0.041	637.38 ±18.69	637.4 ±18.7	423.758 ±12.834
P. albonotatus	7	1.009 ±0.071	--	0.229 ±0.043	0.656 ±0.089	–163.3 ±11.4	0.959 ±0.013	638.11 ±11.81	2670.4 ±154.7	527.644 ±7.889
P. biligonigerus	6	0.898 ±0.057	--	0.361 ±0.034	0.533 ±0.137	–297.0 ±14.7	0.624 ±0.146	528.97 ±53.36	1957.2 ±179.8	329.59 ±30.72
P. enesefae	7	0.703 ±0.050	--	0.265*	0.260 ±0.012	–186.5 ±24.9	0.068 ±0.019	453.01 ±15.21	878.1 ±39.8	359.33 ±18.281
P. signifer	7	0.334 ±0.007	--	1.163 ±0.082	0.261 ±0.010	15.4 ±9.3	0.044 ±0.026	362.99 ±13.64	1159.7 ±55.3	350.666 ±3.979

*From Tárano (2001).

Table 5.6. Comparisons of call differentiation between allopatric and sympatric species pairs. Acoustic characters were collapsed with Principal Component Analyses and the resulting PC scores were used to calculate pairwise species (observed) distances. In the phylogeny-based test, the difference between sympatric and allopatric distances was compared to a null distribution of differences generated from 500 simulations of a character evolving along the phylogeny, under a Brownian-motion model. In the randomization test, the observed distances were compared to a null distribution generated from 10,000 random assignments of species pairs distances to the allopatric and sympatric categories. See text for details.

Variable	*P* Phylogeny-based test	*P* Randomization test
PC I GLOBAL	0.310	0.859
PC II GLOBAL	0.142	0.064
PC III GLOBAL	0.792	0.299

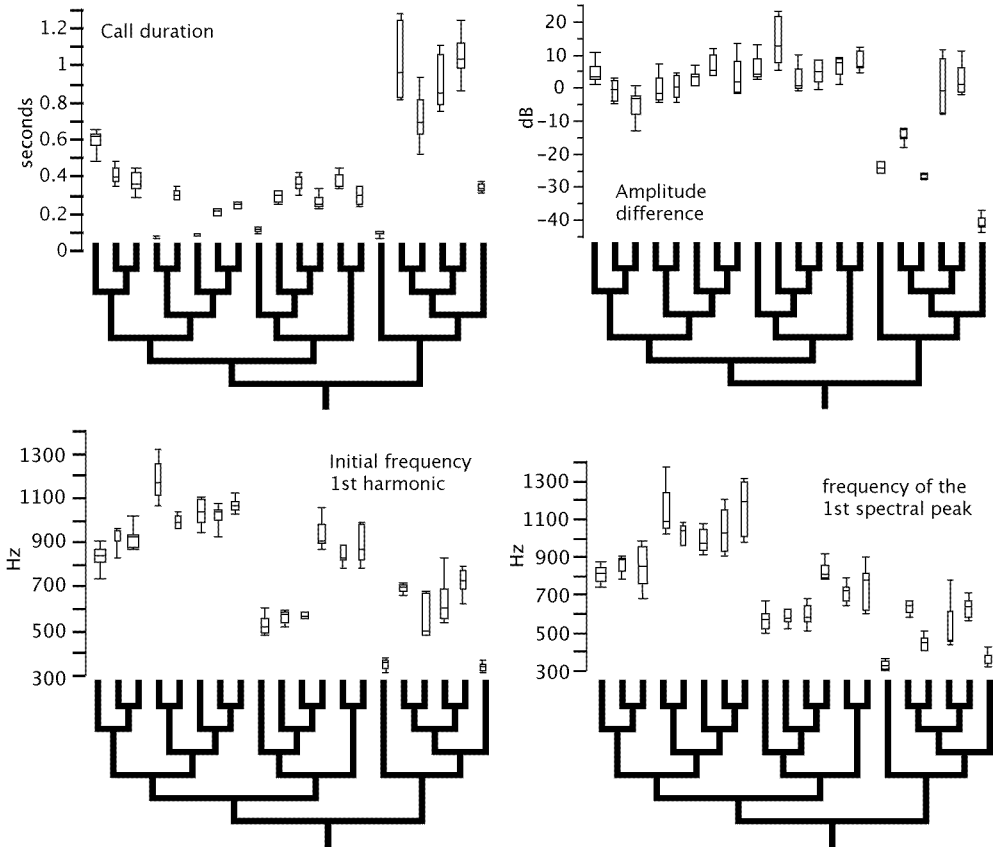

Figure 5.1. Boxplots for characters from advertisement calls. Phylogeny is based on Ron (2006). From left to right, taxa are: *E. pustulatus*, *E.* sp. B, *E.* sp. D, *E. guayaco*, *E. coloradorum*, *E. montubio*, *E. randi* (S), *E. randi* (N), *E. freibergi*, *E. petersi* Puyo, *E. petersi* Yasuní, *E. petersi* Selva, *E. pustulosus* (E), *E. pustulosus* (W), *Eupemphix nattereri*, *Physalaemus albonotatus*, *P. enesefae*, *P. biligonigerus*, *P. barrioi*, and *P. signifer*.

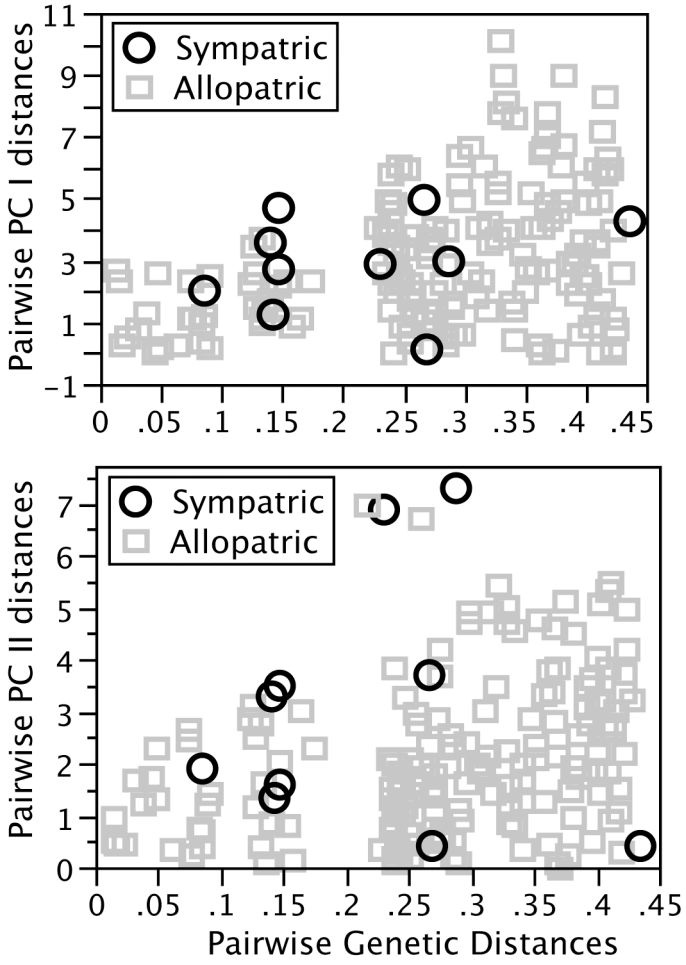

Figure 5.2. Call distance (vertical axes) vs. genetic distance between species pairs of *Engystomops* and six outgroup taxa. Squares and circles indicate allopatric and sympatric species pairs, respectively. Call distances correspond to PC I and PC II from Principal Components Analysis based on 14 acoustic characters (GLOBAL PCA). Genetic distances are maximum likelihood distances under the model GTR + Γ + I, using the same model parameters reported in Ron et al. 2006.

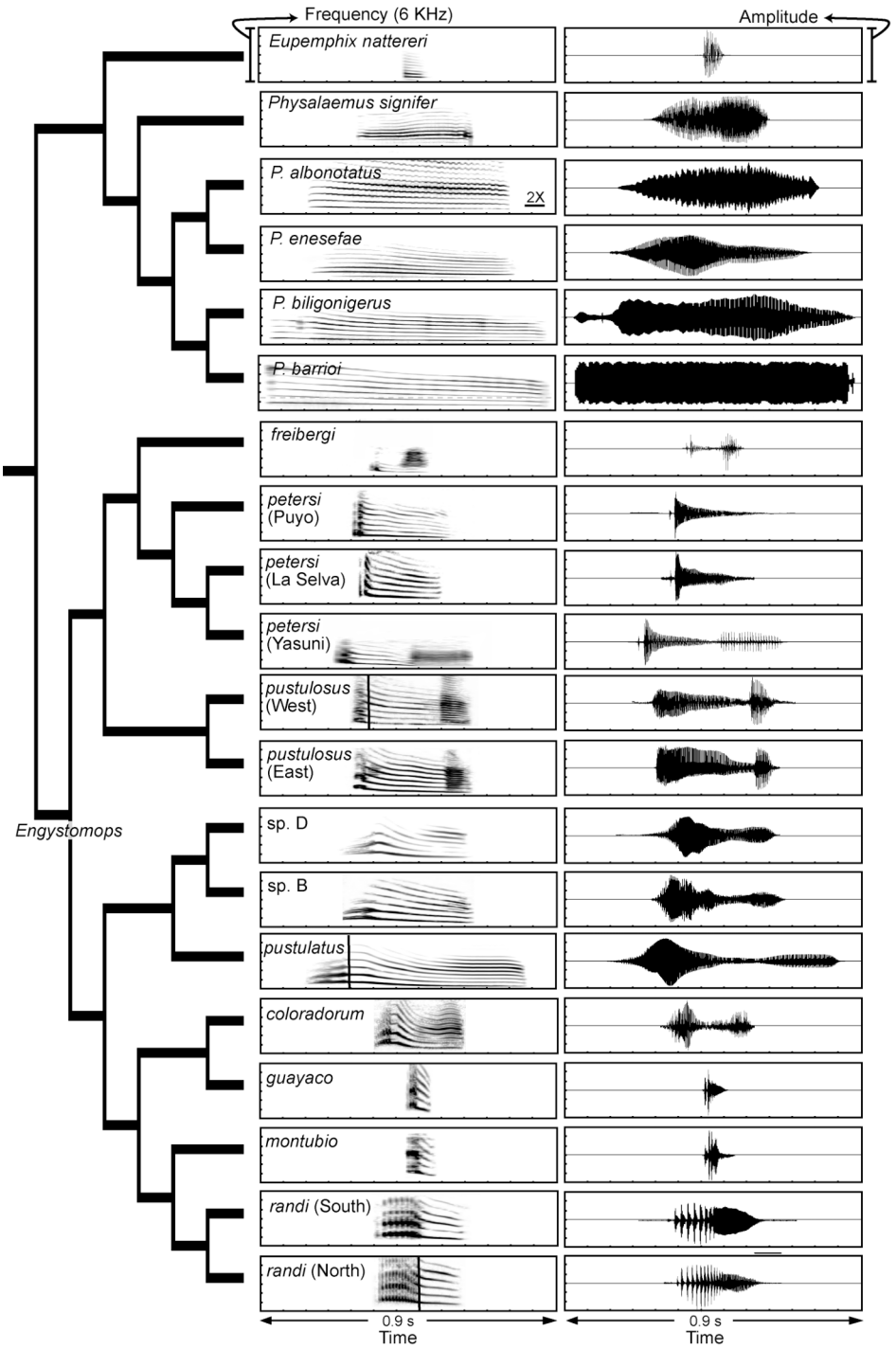

Figure 5.3. Spectrograms (left) and oscillograms (right) of the advertisement calls of *Engystomops* and six outgroup taxa (phylogeny from Ron et al., 2006). In *Engystomops*, the call consists of a downward frequency sweep anteceded by an amplitude-modulated prefix (as an example, the limit between them is shown with a black line in *E. pustulosus*, *E. pustulatus*, and *E. randi*). The vertical axis of all spectrograms ranges from 0 to 6 KHz. In all species but *Physalaemus albonotatus*, the horizontal axes represent 0.9 s (*P. albonotatus* = 1.8 s).

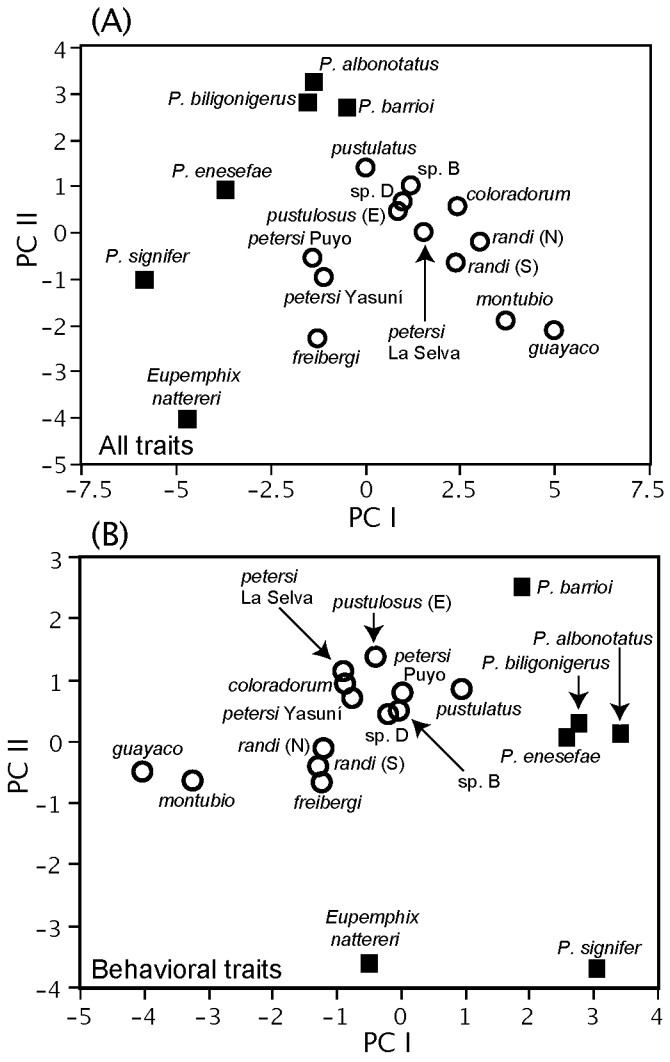

Figure 5.4. Axes I and II from Principal Components Analysis based on acoustic characters of advertisement calls: (A) Including all acoustic characters; (B) Including only characters driven by behavior or physiology. Circles refer to *Engystomops*; squares to *Physalaemus*.

Supplemental Data 5.1. Locality data and voucher information for recordings used in call analyses. QCAZ = Museo Zoología Pontificia Universidad Católica del Ecuador, WCF = W. C. Funk field series, SC = QCAZ field series. The number of individuals analyzed is shown in parenthesis.

E. coloradorum (*n* = 5): Ecuador: Pichincha: Tinalandia, QCAZ 28680, 28682; recorded by S. R. Ron.

E. freibergi (*n* = 7): Peru: Madre de Dios: Tambopata, WCF 2366–67, 2409, 2432, 2451, 2462, 2504; recorded by W. C. Funk.

E. guayaco (*n* = 7): Ecuador: Guayas: Cerro Masvale, QCAZ 19751, 19561–62, 23508, 23510, 23512, 23516; recorded by S. R. Ron.

E. montubio (*n* = 7): Ecuador: Manabí: Puerto Rico, QCAZ 19516–17, 19519, 19520, 19550, 19552, 19555; recorded by D. C. Cannatella, and S. R. Ron.

E. petersi (La Selva; *n* = 7): Ecuador: Sucumbíos: Hostería La Selva, WCF 2643, 2648–49, 2664–67; recorded by W. C. Funk.

E. petersi (Puyo; *n* = 7)*:* Ecuador: Pastaza: El Puyo, QCAZ 26249, 26252, 26254, 26257–58, 26263, 26266; recorded by S. R. Ron.

E. petersi (Yasuní; *n* = 7): Ecuador: Orellana: Estación Científica Yasuní, Universidad San Francisco de Quito, WCF 2686, 2688; recorded by W. C. Funk. Orellana: Estación Científica Yasuní, Universidad Católica; recorded by K. E. Boul.

E. pustulatus (*n* = 10): Ecuador: Cotopaxi: La Maná, QCAZ 26121–22, 26519, 26651, 26708–09, 26723–24, 26774, 26786; recorded by S. R. Ron.

E. pustulosus (E) (*n* = 7): Panama: Panama: Gamboa; recorded by X. Bernal.

E. pustulosus (W) (*n* = 7): México: Chiapas: Tehuantepec; recorded by M. J. Ryan.

E. randi (N) (*n* = 7): Ecuador: Guayas: Cerro Masvale, QCAZ 19559–60, 19563–64, 19565–66, 19753; recorded by D. C. Cannatella and S. R. Ron.

E. randi (S) (*n* = 7): Ecuador: El Oro: Pasaje, QCAZ 19595, 19597–99, 19600, 19602–03; recorded by D. C. Cannatella and S. R. Ron.

E. sp. B (*n* = 7)

Peru: Piura: Moropón, SC 16045–47, 16049, 16051, 16052–53, recorded by D. C. Cannatella and S. R. Ron.

E. sp. D (*n* = 10): Ecuador: El Oro: Puyango, QCAZ 2648385, 26487, 26968–971, 26978, 27016; recorded by S. R. Ron.

E. nattereri (*n* = 7): Brazil: Mato Grosso: Municipio Caceres: recorded by A. Cardoso, A. S. Rand.

P. albonotatus (*n* = 7): Brazil: Mato Grosso: Municipio Caceres: recorded by A. Cardoso.

P. barrioi (*n* = 7): Brazil, Parque "Ibitapioca", Cerro do Bocana; recorded by A. S. Rand.

P. biligonigerus (*n* = 6): Brazil: "Osario"; recorded by A. Cardoso.

P. enesefae (*n* = 7): Venezuela: Guarico: 50 km S from Calabozo; recorded by Z. Tárano.

P. signifer (*n* = 7): Brazil: Río de Janeiro: Horto Forestal Santa Cruz; recorded by A. Cardoso, A. S. Rand.

Supplemental Data 5.2. Pattern of distribution of phylogenetic signal in sexually selected traits. Studies targeted to communities instead of clades were excluded. The type of evidence used to assess phylogenetic signal is listed. "Phylogenetic congruence" refers to support or conflict between traits and an independently derived phylogeny. "Correlation with phylogeny" refers to quantitative measurements of the correlation between trait divergence and lineage divergence.

Taxon	Type of trait	Phylogenetic signal	Type of evidence	Source
Oropéndolas birds	Vocalizations	Strong	Phylogenetic congruence	Price and Lanyon, 2002
Kinglets birds	Vocalizations	Strong	Phylogenetic congruence	Päckert et al., 2003
Bufo and *Pseudacris*	Vocalizations	Strong	Phylogenetic congruence	Cocroft and Ryan, 1995
Túngara frogs	Vocalizations	Strong	Correlation with phylogeny	This study
Wood warblers	Vocalizations	Intermediate	Correlation with genetic distance	Van Buskirk, 1997
Phylloscopus birds	Vocalizations	Strong	Phylogenetic congruence	Helbig et al., 1996
Manakin birds	Lek displays (vocal and visual)	Strong	Phylogenetic congruence	Prum, 1997
Cyclura iguanas	Headbob displays	Weak	Character reconstruction	Martins and Lamont, 1998
Sceloporus lizards	Headbob displays	Weak	Correlation with phylogeny	Martins, 1993; Martins et al., 2004

Taxon	Type of trait	Phylogenetic signal	Type of evidence	Source
Liolaemus lizards	Headbob displays	Weak	Correlation with phylogeny	Martins et al., 2004
Anole lizards	Headbob and dewlap displays	Weak	Correlation with phylogeny	Ord and Martins, 2006
Birds	Sexual dichromatism	Weak	Correlation with phylogeny	Phillimore et al., 2006
Bowerbirds	Bower architecture	Weak	Phylogenetic congruence	Kusmierksi et al., 1997
Storks	Courtship displays	Strong	Phylogenetic congruence	Slikas, 1998
Laupala crickets	Courtship song	Weak	Phylogenetic congruence	Shaw, 1993; Shaw, 1996
Lacewings	Courtship song	Weak	Phylogenetic congruence	Henry et al., 1999
Psylloidea Hemipterans	Courtship song	Strong	Phylogenetic congruence	Percy et al., 2006
Drosophila repleta group	Courtship song	Intermediate	Phylogenetic congruence	Ewing, 1986
Drosophila willistoni group	Courtship song	Weak	Phylogenetic congruence, Correlation with genetic distance	Gleason and Ritchie, 1998

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